THE MYOCARDIUM—
ITS BIOCHEMISTRY
and
BIOPHYSICS

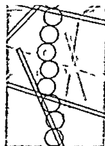
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Guest Editor

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The symbols represent a diagram of an actomyosin swarm by Wilfried F H M Mommaerts

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* Deceased May 9 1960



THE MYOCARDIUM—ITS BIOCHEMISTRY and BIOPHYSICS

Foreword

IT IS TRADITIONAL both in the physiologic laboratory and in the clinic, to regard the heart as a muscular pump. Moreover, in appraising the performance of this pump, it has become customary to assess separately the work of the right and of the left ventricles, a practice that involves the measurement of the blood pressure developed and the volume of blood ejected by each ventricle during systole. From this hemodynamic approach has come not only the broad generalizations about the behavior of the heart, i.e., "Starling's Law," "Bowditch's All or None Law," and the "Staircase Phenomenon," but also the criteria for estimating the capacity of each ventricle for performing work as well as its adaptability to different work loads.

However, as it becomes clear that current hemodynamic measurements per se promise fewer and fewer broad generalizations, attention is turning to the biochemical and biophysical aspects of heart muscle. The heart is being scrutinized as a biologic engine for transducing the chemical energy of metabolism into useful mechanical work. Leading and directing this new approach are the electron microscopists, the biochemists and the biophysicists who bring lessons learned from muscular and nonmuscular contractile tissues to the study of the myocardium.

*Their first studies have succeeded in illu*minating certain features of myocardial function. But these studies have also emphasized how little we know about the coordinated performance of the heart. Indeed, in the sphere of coordinated behavior, they have raised more questions than they have answered. How does the myocardial engine work? How is the chemical energy converted into mechanical energy? Is there a general principle of contractility and how does the myocardium illustrate it? What sets the contractile process into motion? What is the molecular basis for electric-chemical-mechanical coupling?

These questions about the coordinated behavior of the heart muscle should not obscure the wealth of observations and experiments that preceded them. Muscle has probably been studied more intensively than any other tissue. Indeed, such questions could not be posed were it not for a host of antecedant observations and experiments. Unfortunately, only the future can disclose if the proper observations were made, if the proper experiments were performed, and if the proper questions are being asked.

Although this conference is deeply rooted in classical physiology, nearly all of its topics are products of modern times. This point is easily illustrated with respect to the energy for muscular contraction. Thus, only in 1907 did Fletcher and Hopkins disclose that lactic acid is formed when muscle contracts. By the 1920s, the time was ripe for Hill, Meyerhof *and Fenn to consider, in a systematic fashion* the energy liberated by a contracting muscle. By 1930, it was clear that (1) myosin is part of the contractile machinery of muscle

(2) the energy expended during contraction derives from the breakdown of glycogen into lactic acid and (3) creatine phosphate and ATP are present in muscle During the 1930s the role of creatine phosphate was elucidated and parallels were drawn between glycolysis in muscle and alcoholic fermentation Not until the 1940s was it shown that (1) myosin catalyzed the splitting of ATP (2) actin and myosin were separable and (3) the contractile behavior of muscle could be mimicked in vitro In the same decade the electron microscope was also first applied to the visualization of the ultrastructure of heart muscle In the 1950s and 1960s attention has been focused on the intracellular molecular sources of energy for contraction and on models that are designed to account for the behavior of contracting muscle in vivo

This outline of a steady progression from larger to smaller units has skipped the uncertainties that haunt the isolation of cellular ingredients from their natural surroundings Is the final preparation typical of living muscle or is it an artifact of synthesis? What does the behavior of the contractile thread or the isolated sarcomere mean for the behavior of intact muscle? Mephistopheles recognized that to comprehend a living thing past any doubts it is necessary to cancel first the living spirit out But how does the final analysis take into account the living link you banned ? How relevant is the behavior of skeletal or smooth muscle to the behavior of heart muscle? Is there a general law of contractility that is epitomized by the behavior of heart muscle? How meaningful are the ingenious anatomic and physicochemical models for the understanding of living muscle?

The time is not yet ripe for answers to many or possibly to any of these questions Nor was this meeting arranged to provide such answers Instead the meeting has more circumscribed goals to set questions that await solution alongside of the observations that have led to their formulation to encourage investigators to illuminate boundaries between different interests so that these boundaries may be crossed to allow the uninitiated to peer at muscle through the disciplined eyes of scientists devoted to the study of muscle and hopefully to help to shape a question or two that might otherwise have remained amorphous

ALFRED P FISHMAN M D
Guest Editor

Muscle as a Contractile Tissue

it must not be supposed that muscles operate by any unique mechanism not represented in other contractile tissues The fundamental mechanism of contraction is presumably the same in all tissues but in muscles it is less obscured by other functions such as digestion absorption and excretion and it is easier to measure the forces developed easier to observe the physical changes which occur and easier to determine the chemical nature and quantities of the reactants and end products of the chemical processes involved —W O Fenn *Section on Contractility* in R Hober *Physical Chemistry of Cell and Tissues* Philadelphia Blakiston 1945 p 447

I Ultrastructure

Chairman Charles E Kossmann M D

On Smallness

By CHARLES E KOSSMANN M D[1]

BY WAY OF INTRODUCTION to the material on Ultrastructure to be presented in this initial portion of the Symposium on the Myocardium a few remarks on *the general problem of Smallness are in order*

When one probes a little into the scientific realm of the previously unseen some paradoxes and apparent inconsistencies are encountered that make comprehension by the uninitiated a little difficult I will attempt to share with you 1 or 2 of the personal problems encountered in learning about this *strange new world* which I hope may implement your own understanding of the exciting presentations about to be made

We might begin with the word Ultrastructure itself ' Ultra is a prefix that, in the Latin from which it is borrowed means beyond ' ' Beyond structure by itself is relatively meaningless but perhaps those who coined the word had in mind a structure beyond something That something is visibility and for purposes of definition visibil*ity even when augmented with a compound* light microscope

Ultrastructure then is really a contraction of ultramicroscopic structure" meaning that it is substance of such small size as to be beyond that which can be seen with a light microscope Despite the definition a microscope is indeed used But it is a special type available for barely 2 decades but nevertheless now quite familiar to most morphologists It is the electron microscope[1]

Human curiosity naturally prompts one to ask [illegible] cope differs from an ordi[illegible] e Both utilize the refr[illegible] n [illegible] one the refracted bea[illegible] t [illegible] r a stream of elec*tro*[illegible] W[illegible] th[illegible] rs involved are investi[illegible] fu[illegible] a singularly surprising disc[illegible] is [illegible] o the effect that in the field of [illegible] a dualism exists which on th[illegible] rf[illegible] least appears to be a paradox The el[illegible] tron is regarded not *only* as a particle [illegible] al o as a *wave* Further and mo t im[illegible] t from the microscopist s *point of view the* wave has a length that is only a fraction of the length of an ordinary light wave Basically it is this difference in wave lengths that accounts for the different resolving powers of the 2 types of microscopes under discussion For the light microscope the limit of resolution for tissues is in the neighborhood of 0 25 micron of an electron *microscope 20 to 30 A Under ideal con*ditions then the resolving power of the latter is 100 times greater than of the former In *the biologic* sciences it is the new anatomic realm revealed by this superior performance of the electron microscope that constitutes what is now known as Ultrastructure

It might be appropriate at this point to recall the measurements used in defining degrees of smallness Justification for repeating these elementary measurements is the pre*sumption that most of you do* not use the electron microscope in your everyday activities

$$1 \text{ mm} = 1\,000\ \mu = 10\,000\,000\ \text{A}$$

Another way of saying this is that

$$0\,001 \text{ mm} = \mu = 10\,000\ \text{A}$$

From New York University School of Medicine New York New York.

By way of a minor but purposeful digression if the problems of wave mechanics and theory as applied to the electron microscope are studied it is learned that the electrons in the beam being particles have energy defined by Einstein's well known relation for the equivalence of mass and energy namely

$$E = mc$$

The equation has relevance to more than a beam of electrons To the physicist it demonstrates among other things the important concept of variation of mass with velocity To the biologist it suggests, if it is correct that eventually the division between structure and function must become narrow indeed As this symposium wends on all of us will probably become aware of the considerable decrease in the gap between structure and function that has resulted from the original investigations about to be presented

In considering the degrees of smallness with which our distinguished speakers on Ultrastructure have concerned themselves one wonders what will be the next order of magnitude to be studied It is obvious, for example that pores exist in membranes which cannot be seen even with the electron microscope What will be the eventual extreme of visible smallness? How small is infinitely small? What is the limit of smallness of which man s mind can conceive? In such frustrating questions as these can be recognized the substrate for a new type of neurosis among ultramicroscopist and perhaps among all biologists But this theoretical occupational disease need not concern us at present There is too much yet to be learned about ultrastructure now visible and it is about this as found in the myocardium that we will hear this morning

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One Life

Four decades of research have left no doubt in the author s mind that there is only one life and one living matter however different its structure colorful its functions and varied its appearance We are all but recent leaves on the same old tree of life and even if this life has adapted itself to new functions and conditions it uses the same old basic principles over and over again There is no real difference between the grass and he who mows it The muscles which move the mower need the very same two substances for their motion as the grass needs for its growth potassium and phosphate the two substances we put on our lawn as fertilizer so as to have something to mow—a strikingly simple demonstration of the basic unity of living Nature —A Szent Gyorgyi *Chemistry of Muscular Contraction* Ed 2 New York Academic Press Inc 1951 p 5

The Contractile Structure of Cardiac and Skeletal Muscle

By H E Huxley, Ph D

Previous findings that have led to the sliding filament model for striated muscle are reviewed together with some recent observations on isolated filaments produced by a new procedure The basic structure of the contractile apparatus in skeletal and in cardiac muscle appears to be identical The relation between certain special features of cardiac muscle and its structure is discussed

I SHOULD LIKE first of all to review very briefly the picture we now have of the fine structure of striated muscle This will be very familiar to many people here but I hope to mention one or two new pieces of evidence to maintain their interest It will be useful to set out this picture again I believe since many of its features are established with a rather high degree of certainty and have been accepted by its original opponents it is therefore likely to be both profitable and fairly correct to think of muscles in these terms when trying to explain their various properties The features I shall describe appear to be common to both cardiac and skeletal muscle

Review of Structural Observations

The starting point in describing the fine structure is the well known appearance of the cross striations in these muscles illustrated in figure 1 This shows the characteristic alternation of dark and light bands along each of the myofibrils the dense A bands and the less dense I bands The I bands are bisected by the dense Z line and the A bands often show a less dense zone in the center known as the H zone The myofibrils are also composed of longitudinal filaments and when very thin sections are examined (fig 2), it becomes evident that it is the arrangement of the filaments that gives rise to the pattern of striations as Dr Jean Hanson and I suggested on the basis of light microscope observations and the earlier electron micrographs [1] There are 2 types of filaments present, organized into a series of overlapping arrays along each fibril arrays of thicker filaments forming the A bands and arrays of thinner filaments being present in the I bands The thinner filaments extend into the A bands but at rest length do not quite reach to the center leaving there as a result the somewhat less dense H zone The thick filaments have a diameter of about 100 Å and lie in a hexagonal array about 450 Å apart they are each about 1 5 microns long the length of the A band The thin filaments are about 50 Å in diameter and extend approximately 1 micron on either side of the Z line At resting length each sarcomere (Z to Z) is about 2 3 microns long so the width of the H zone is approximately 0 3 micron

Cross bridges extend between the thick and thin filaments Each thin filament is connected to each of its 3 neighboring thick filaments by a bridge every 400 Å along the length of the overlap region giving it a total of about 54 bridges at resting length The total number of bridges in 1 cc of muscle is of the order of 5×10^{16} It is very plausible to suggest that the bridges provide a means by which chemical and mechanical interaction can take place between the arrays of filaments

Muscle fibrils can be disintegrated mechanically in the presence of agents that weaken the forces of attachment of the cross bridges When this is done the structure breaks down into (1) isolated thick (100 Å diameter) filaments nearly always 1 5 microns in length showing projections reminiscent of the cross bridges seen in intact muscle

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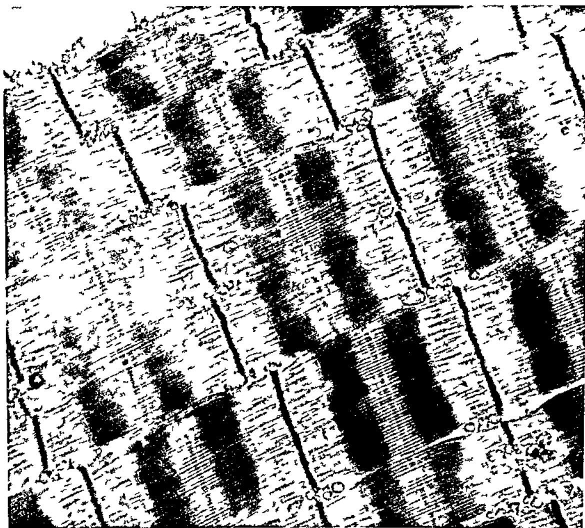


Figure 1

Low magnification electron micrograph of thicker section of rabbit psoas muscle showing a number of myofibrils near the edge of a fiber. A band dense; I bands less dense and bisected by Z line. × [illegible]7,000

(2) isolated thin (50 Å diameter) filaments sometimes 1 micron long and sometimes apparently broken into smaller lengths (3) groups of thin filaments still joined onto a Z line forming an I segment about 2.0 microns in length and (4) occasional groups of thin and thick filaments lying side by side and seemingly joined together by cross bridges. These various structures are illustrated in figures 3, 4 and 5; their appearance provides a new form of confirmation of our previous conclusions. They also provide a new type of experimental material both for electron microscopy and perhaps for biochemical studies also.

Now let us consider the composition of the filaments. At present there are very strong reasons for believing that the thick filaments contain all the protein myosin that is present in the muscle, while the second principal structural protein, actin, occurs in the thin filaments. Solutions known to dissolve out myosin from the muscle selectively



Figure 2

High magnification picture of thin section of rabbit psoas muscle showing arrangement of thick and thin filaments × 150 000

will dissolve out the thick filaments[1,2] leaving the array of thin filaments behind a process that can be observed in the light microscope as the disappearance of the dense A bands Subsequent extraction of actin dissolves away most of the material of the thin filaments These observations have been put on a quantitative basis by the use of the interference microscope[3,4] The amount of material in the A bands arising from the presence of the thick filaments very closely approximates the amount of myosin present and the amount of material removed from the A bands is quantitatively equal to the amount of myosin that could be extracted from the same type of preparation by large scale biochemical technics More recently Perry and Corsi have shown that the selective removal of actin and tropomyosin removes the I band material leaving the A bands (and all the ATPase activity and hence presumably the myosin) intact

The changes that take place in this structure during changes in the length of the muscle can be investigated by light microscope observations as changes in band pattern These show[6,7] that during stretch and during shortening, active or passive the two sets of filaments slide past each other there being no substantial overall change in the length of any of the filaments until it is brought about by steric factors in more pronounced degrees of shortening (e g when sarcomere length decreases below the length of the A bands) When the actin filaments have come together in the center of the sarcomere further shortening seems to cause them to slide past each other giving the double overlap effect illustrated in figure 6

These observations and others lead us to think that the system must function in the following way In the resting state the cross bridges which are projecting parts of the myosin filaments do not attach to the actin filaments which are therefore free to

slide past them easily This accounts for *the high extensibility and relative plasticity* of resting muscle When the muscle is active the cross bridges can attach to specific sites on the actin filaments for a brief period of time during which a relative force and if the muscle is allowed to shorten a relative movement are generated between the 2 types of filament in some way at present unknown The bridge then detaches and is free to form another attachment further along the actin filament if movement has taken place Each bridge will perform a number of such cycles while the muscle is active (about 5 during a single twitch) the energy required for the process being liberated by the splitting of the substrate (probably ATP) by the ATPase of the myosin When activity is over the bridges cease to attach to the actin enzyme activity comes to an end and the muscle returns to the resting relaxed state When the muscle passes into rigor (a condition characterized by the absence of ATP) the cross-bridges become permanently attached to the actin filaments and the muscle is rigid and inextensible for the filaments are not able to slide past each other

Important Features of the Model

Reviews of the large amount of biochemical and physiologic data available concerning striated muscle and the structural model that has been described have already been published in extenso [8, 9, 10] Here we will mention briefly only 3 particular points that seem worth while to emphasize

1 The system can develop a range of different tensions depending upon the number of cross bridges that are active simultaneously For a given load the system shortens with a particular velocity of shortening (rather than say a particular acceleration) such that the number of bridges actively developing tension is just sufficient to bear the load The rate-limiting factor in the system is the rate at which unattached bridges can become attached again and develop tension as a given active site on the



Figure 3

Isolated thick filaments 1 5μ in length prepared by blending glycerinated muscle in the presence of a relaxing agent (EDTA + ATP) Specimen prepared for electron microscopy by negative staining technic × 50 000

actin moves past the bridge there is a certain length of time available for this process to take place the faster the movement the less the chance of attachment to a particular site and the lower the tension at any given moment of time Thus when shortening under a particular load the system will settle down to an equilibrium velocity at which the rate of formation of new links is just equal to the rate of opening of formed links that have already exerted their pull on the actin filament

2 Energy release by the enzyme site is activated by the attachment of the cross bridges to the actin filament Thus the

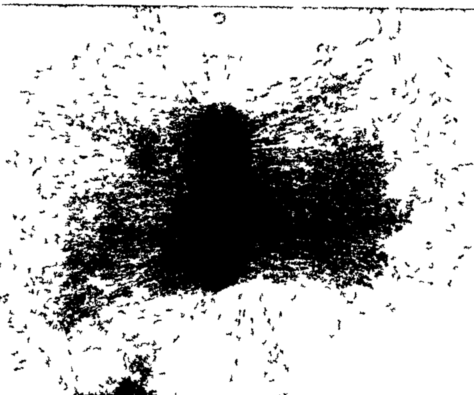


Figure 4

Isolated I segment (array of thin filaments) prepared as in figure 3 × 60 000

number of fully active sites is controlled by the tension in the muscle, and the rate at which those sites repeat their cycles of activity is controlled by the velocity of shortening The system therefore has the possibility of behaving economically, i e releasing more energy for a given distance of shortening when it is shortening against a larger load This is a very important feature of the behavior of real muscles One can see in a general way how such a system might give rise to the type of behavior characterized by the Hill equation

$$(P + a)V = (P_0 - P)b$$

where P = actual load P_0 = isometric tension V = velocity of shortening a = constant (heat of shortening) and b = constant The left side of this equation gives us the total rate of energy release required to do external work and produce shortening heat (assuming that maintenance heat can be accounted for separately) The right side contains the term $(P_0 - P)$ which is proportional to the number of *unattached* bridges If the attachment of such bridges is the rate limiting step as we have assumed then the model could quite naturally give rise to this equation A more elegant and complete account of the mechanical and thermal properties of striated muscle in terms of a particular version of the sliding filament model has been given by A F Huxley[9]

3 It is not necessary to postulate that each cross bridge generates a pull over a distance comparable to that separating successive cross bridges between a given pair of thick and thin filaments or between successive active sites on the actin filaments All that is necessary is that the actin filament shall be drawn along such a distance *between* 2 successive operations of a given cross bridge and this movement can equally well be achieved by small movements—say of 5 Å—produced successively at 10 other cross bridges As any given actin filament has

about 54 bridges directed toward it in each half of the A band, this can be achieved quite easily still leaving the possibility for up to 5 bridges to be acting in parallel at any given moment to permit the variation in tension and rate of energy release with velocity of shortening that we have already described

Special Features of Cardiac Muscle

Although the essential features of the contractile structure and its behavior appear to be the same in cardiac and skeletal muscles there are a few points at which differences occur that may be significant Probably the most obvious one is the presence of very large numbers of mitochondria in cardiac muscles *as compared to skeletal muscle* no doubt associated with the ability of the heart to function continuously over very long period of time without intervals for recovery

The second feature is the comparatively small diameter of heart muscle fibers (as small as a few microns) compared with those of skeletal muscle which are most commonly 50 to 100 microns in diameter The latter are usually provided with quite an abundant reticulum i e a system of internal membranes in each fiber and these are believed to be concerned in relaying the signal for contraction into the interior Such a reticulum is either very sparse or seemingly absent in many of the heart muscle preparations that have been examined a point of difference that in view of the apparently different membrane properties of cardiac muscle deserves further study

Another rather puzzling feature of cardiac muscle is the relatively low tension per unit area which it will develop only about one tenth that of skeletal muscle according to 2 recent studies[11 1-] Some of the difference may be accounted for by the greater fraction of the cross sectional area occupied by mitochondria in the cardiac muscle—perhaps a factor of 2 difference might occur for this reason—but a large factor still remains and there is no obvious reason from the visible structure to account for it It may of course result from different enzymatic properties of cardiac actomyosin This is a difficult hypothesis to investigate, as the enzymatic properties of all actomyosins seem to be rather low in comparison with what one would anticipate from the maximum energy output of the muscles from which they were obtained



Figure 5

Isolated group of thin and thick filaments prepared as in figure 3 negatively stained × 300 000



Figure 6
Heavily contracted muscle showing double overlap of thin filaments × 150 000

A fourth feature of cardiac muscle that distinguishes it from skeletal muscle is the nature of the active isometric length tension curve i e that showing the increase in tension over resting tension when the muscle i active In skeletal muscle this curve exhibits a maximum around resting length (which lies very close to the greatest length at which the muscle develops zero resting tension) As the length of the muscle is increased beyond resting length the active tension decreases an effect that has been explained by Huxley and Niedergerke[6] as resulting from the decreased length of the region in which actin and myosin filaments overlap and can form cross links with each other The factors that cause the tension to decrease below resting length are unknown In cardiac muscle however the active tension *increases* as the length of the muscle is increased beyond resting length as defined above and reaches a peak only after a stretch of about 30 per cent[12] This effect might be explained if the sarcomeres of cardiac muscle at resting length resembled either in the extent of overlap or in the factors that produce the decrease in tension at length below rest length those of skeletal muscle that had shortened by about 25 per cent

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The Sarcoplasmic Reticulum of Skeletal and Cardiac Muscle

By DON W FAWCETT MD

This paper traces the development of our present concept of the structural organization of the sarcoplasmic reticulum in striated muscle and reviews the physiologic evidence for its participation in intracellular impulse conduction Comparative observations are presented showing that this system of membrane limited tubules is particularly well developed in exceptionally fast acting skeletal muscles These findings are interpreted as evidence supporting the hypothesis that the reticulum is involved in the coupling of excitation to contraction but it is considered likely that it also has other important functions in muscle metabolism The sarcoplasmic reticulum of cardiac muscle is found to be much less extensive and less precisely arranged in relation to the cross banded pattern of the myofibrils than it is in skeletal muscle It is believed nevertheless that it may prove to have a significant role in the physiology of the myocardium

AMONG THE MOST SIGNIFICANT recent morphologic contributions to our understanding of muscle have been the demonstration by Huxley and Hanson[1] that the actin and myosin of the myofibrils form two distinct sets of interdigitating filaments, and the description by Bennett[2] and Porter and Palade[3] of the *sarcoplasmic reticulum* a submicroscopic plexiform system of membrane bounded tubules that occupies the interfibrillar spaces throughout the muscle fiber The first of these discoveries has formed the basis for a new and now widely accepted sliding filament theory of muscle contraction and the second has defined a new organelle in the sarcoplasm that may play an important role in the coupling of excitation to contraction

We propose to review the evidence for the current belief that the sarcoplasmic reticulum may be involved in intracellular impulse conduction and then to present some comparative observations on the organization of this system of membranes in certain examples of skeletal and cardiac muscle that have unusual physiologic properties

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This research was supported in part by Research Grant RG 6709 from the U S Public Health Service and by Grant G 12916 from the National Science Foundation.

Historical Considerations

Delicate intracellular networks surrounding the myofibrils were observed over half a century ago by Thin[4] Retzius,[5] Veratti[6] and a few other able cytologists in preparations of muscle stained by special metal impregnation methods However the membranous nature of this system its continuity throughout the sarcoplasm and its exact relationship to the contractile elements could not be fully appreciated with the light microscope The reticulum therefore aroused the interest of very few morphologists and was quite unknown to physiologists until it was rediscovered a few years ago by Bennett and Porter[7] Andersson[8] and Porter and Palade[3] in electron micrographs of skeletal muscle

In these studies electron micrographs of thin sections passing tangential to the myofibrils often revealed a plexus of smooth surfaced tubules closely applied to their surface From the examination of large numbers of micrographs of *Amblystoma* muscle Porter and Palade arrived at an interpretation of the distribution of the sarcotubules that is presented diagrammatically in figure 1A The tubules overlying the A band of each sarcomere are predominantly longitudinal in their orientation but communicate laterally with one another in the region of the H band At the ends of each sarcomere the longitudi

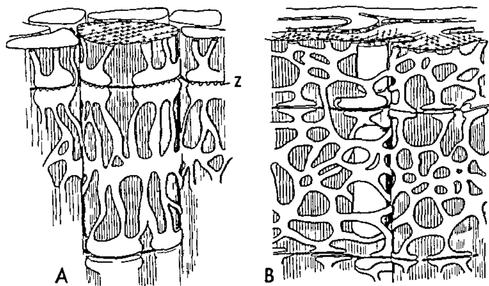


Figure 1

A *Diagrammatic interpretation of the organi ation of the arcoplasmic reticulum in skel tal muscle of the salamander* Amblystoma punctatum *Each myofibril is surrounded by a plexiform system of tubules The tubules overlying the A band are predominantly longitudinal in their orientation but communicate fre ly in the region of the H band At th ends of each sarcomere the longitudinal tubules of the reticulum are confluent with dilated transverse channels call d* terminal cisternae *The complex oriented trans rsely at the Z band consisting of terminal cisternae and an intermediate row of mall vesicles or hort tubul s is referred to as a* triad B *Diagram of the sarcoplasmic reticulum of rat cardiac muscle The loose network of sarcotubes shows le s regional differentiation in relation to the cross banded pattern of the myofibrils The terminal cisternae are small and typical triads are uncommon (From Bennett H S* In *Muscle vol 1 New York Academic Pr ss Inc Redrawn from Porter and Pala le [3])*

nal meshes of the reticulum are confluent with dilated transverse channels called terminal cisternae The terminal cisternae of suc cessive segmental units of the reticulum are situated on either side of the Z band sepa rated by a row of small vesicles The complex consisting of two terminal cisternae and the intermediate row of vesicles is referred to as a triad of the reticulum In *Amblystoma* muscle these are oriented transversely or cir cumferentially with respect to each myofibril and are located on either side of the Z line

This system of sarcoplasmic tubules was interpreted by Porter and Palade as a special form of the *endoplasmic reticulum* an organ elle that they had described earlier in a wide variety of other cell types In muscle how ever the reticulum lacked the ribonucleopro tein particles or ribosomes commonly associ ated with it in glandular cells and it was dis tributed in a very precise relation to the cross banded structure of the myofibrils This organization suggested to several investiga tors[3 8] that the reticulum might have a special role in muscular contraction possibly providing pathways for preferential diffusion of metabolites or intracellular spread of exci tation

Physiologic evidence tending to support this latter speculation was soon provided by the ingenious experiments of Andrew Huxley and Taylor[9] These investigators were con cerned with the intracellular mechanisms whereby contraction of myofibrils deep in the interior of the muscle fiber is coupled to ex citation of the surface membrane In 1948 A V Hill[10] had concluded from considera tion of the rates and distances involved that

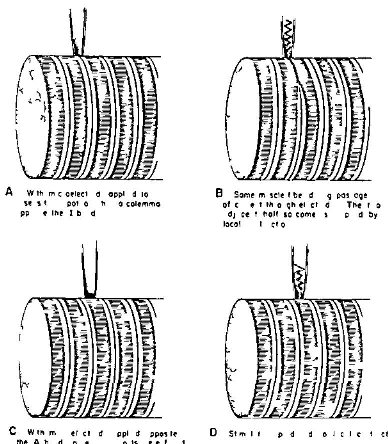


Figure 2

Diagrammatic representation of the experiments that suggested that some structural component located at the I band of frog skeletal muscle was responsible for the inward spread of excitation. Stimulation with the microelectrode over the I band often resulted in contraction of the adjacent half sarcomeres, whereas no contraction resulted from stimulation at the A band. (Drawing based on illustrations in the paper by Huxley and Taylor.[8])

the latency of response in skeletal muscle is much too short to be accounted for by the inward diffusion of a hypothetical activating substance from the sarcolemma to the contractile elements. Approaching this problem with new methods Huxley and Taylor applied a microelectrode to different points on the sarcolemma of single frog muscle fibers under direct observation with an interference microscope (fig. 2). When the tip of the micropipette was over the I band (fig. 2A) passage of current was often followed by contraction of the adjacent half sarcomeres (fig. 2B), but no response was obtained when the stimulus was applied over the A band (figs. 2, C and D). These results suggested that some structural component located in the I band was responsible for the inward spread of excitation. The possibility that it was the Z band itself was considered, but this had to be abandoned when similar experiments on lizard muscle showed that the sensitive spots on the sarcolemma in this species were not at the level of the Z band but over the A band near the A-I junction. Electron microscopic studies on the muscles of these two species revealed that the triads of the sarcoplasmic reticulum are situated at the Z band in frog muscle but at the A-I junction in lizard muscle. The close correspondence between the position of the triads in the reticulum and the level in the sarcomere of spots sensitive to direct stimulation with a microelectrode strongly suggested that the impulse might be conducted inward by the membranes of the sarcoplasmic reticulum. This, then, is the historical background from which our own interest in the sarcoplasmic reticulum developed.

The Sarcoplasmic Reticulum of Fast Acting Skeletal Muscles

If the sarcoplasmic reticulum is involved in the coupling of excitation to contraction

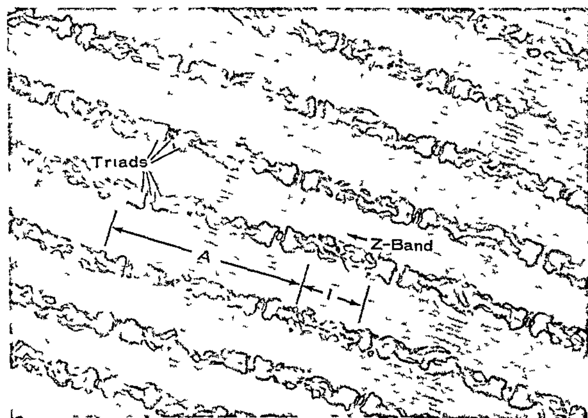


Figure 3

Longitudinal section of the sound producing muscle in the swimbladder of the toadfish Opsanus tau. In this unusually fast acting muscle the sarcoplasmic reticulum is exceptionally well developed. There are 2 triads to each sarcomere length located over the A band near the A I junctions. Longitudinal tubules extend in either direction from each triad toward the H and toward the Z band respectively. The longitudinal elements of the reticulum are continuous over the H band but often appear to be interrupted at the Z band (see at arrows). (Electron micrograph by Dr J P Revel.)

one might expect to find differences in its organization or its degree of development in muscles having different speeds of contraction. With this in mind Dr Jean Paul Revel and I have studied the reticulum of some particularly fast acting muscles.

The first of these is a muscle that forms an equatorial band around the swim bladder of the common toadfish *Opsanus tau*. Rapid contractions of this muscle set up vibrations in the taut gas filled bladder that produce the audible sounds made by these fish when they are courting or when otherwise disturbed. This muscle is said to attain its peak contraction in only 5 to 8 msec[11] and requires some 300 stimuli per second to tetanize.[12] Since the fine structure of this muscle has been described in detail in a separate publication[13] only a brief account of its salient features will be presented here. The fibers are of large diameter. Their myofibrils are flat ribbon like structures arranged radially around a central core of sarcoplasm to form a thick walled contractile cylinder. Mitochondria are seldom found between the myofibrils. Instead they are located either in the core of the contractile cylinder or in the superficial layer of sarcoplasm around its periphery. The narrow clefts between the broad faces of the myofibrils are occupied by a highly developed



Figure 4

Electron micrograph of a thin section passing through toadfish muscle parallel to the broad fa e of a myofibril The triads are seen running across the myofilaments parallel to each other and to the Z band Crowding and superimposition tend to obscure the plexiform nature of the reticulum between the successive triads The middle element of the triad which appears here as a row of small vesicles is actually a continuous slender tubule

sarcoplasmic reticulum The ribbon like myo fibrils present their narrowest dimension in a longitudinal section through the wall of the contractile cylinder (fig 3) There is a long A band and a rather short I band and these are precisely aligned across the entire width of the fiber The reticulum in the interfibrillar clefts is extremely regular in its organization and shows two triads in each sarcomere length These are invariably located near the A I junctions where the two interdigitating sets of filaments described by Hugh Huxley[14] are presumed to slide with respect to one another during muscular contraction The triads run transversely across the broad face of the myo fibrils and radially with respect to the con tractile cylinder as a whole Thus in most longitudinal sections of the muscle fibers the triads are seen in cross section (fig 3) Each consists of a slender intermediate tube ap proximately 30 mμ in diameter flanked by two larger cisternae about 110 mμ across The longitudinal tubules connecting succes sive triads run parallel to the myofibrils and anastomose freely to form reticula in two or more layers that are closely applied to the surfaces of the adjacent contractile elements

Sections passing through the interfibrillar clefts in which the triads are cut longitudinal ly as they traverse the broad face of the under lying myofibril provide a more extensive view of the reticulum (fig 4)) In this view the intermediate element often appears as a row of vesicles but in the best preserved speci mens it seems to be a continuous tubule In the intervals between triads the plexiform nature of the longitudinal elements of the re ticulum is often obscured by superimposition



Figure 5

An electron micrograph of a section passing tangential to a myofibril in the cricothyroid muscle of the bat Myotis lucifugus *In this fast acting mammalian muscle too the sarcoplasmic reticulum is far more elaborately developed than in slower muscles The triads are located at the A I junctions and the longitudinal sarcotubules seem to be continuous across the Z band as well as across the H band (Micrograph by Dr J P Revel)*

of more than one layer of sarcotubes so that it is difficult to ascertain whether the reticulum is continuous from sarcomere to sarcomere across the Z band or only between triads within the same sarcomere At the outer margin of the contractile cylinder the cisternal elements of the triad narrow abruptly and follow a sinuous course among the mitochondria in the peripheral layer of sarcoplasm Some of them can be followed to the sarcolemma It is assumed that such points of contact of the reticulum with the surface membrane may correspond to th sensitive points found in frog and lizard muscle with the searching microelectrode in Huxley and Taylor s experiments

Seeking a fast acting mammalian muscle for study Dr Revel in our laboratory has examined the cricothyroid muscle of the bat [15] Physiologic measurements of the time course of contraction in this muscle have not been made, but one can infer from its normal function that it is very fast In its sonic navigation the bat uses pulses of supersonic sound of the order of 5 to 10 msec in duration and within this brief period it is capable of modulating the frequency over a considerable range To accomplish this the cricothyroid must be able to change its state of contraction very rapidly When examined in electron micrographs of low magnification the appearance of this muscle does not differ greatly from other mammalian muscles The mitochondria are numerous and arranged in rows between myofibrils of the usual rounded or polygonal cross sectional shape At higher magnification it is evident that the sarcoplasmic reticulum is exceptionally well developed (fig 5) The transverse triads near the A I junctions appear to encircle the myo-

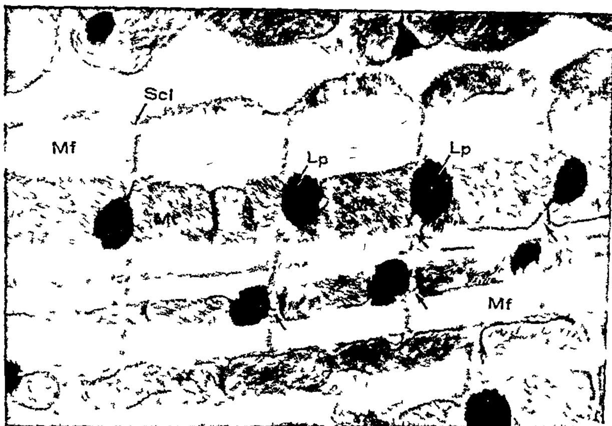


Figure 6

Longitudinal section of a peripheral portion of a cardiac muscle fiber from the bat heart Many large mitochondria (MT) are located immediately beneath the sarcolemma (Scl) and between the myofibrils (MB) Numerous lipid droplets (Lp) among the mitochondria are evidently used as an energy source At several places indicated by arrows transverse elements of the sarcoplasmic reticulum corresponding to the triads of skeletal muscle are seen between the mitochondria at the level of the Z band

fibril completely and are no doubt continuous with the triads of adjacent myofibrils Their cisternae are more slender than those of the fish muscle described earlier and the intermediate element more commonly appears to be a narrow continuous tube The longitudinal sarcotubules of the reticulum are also of smaller and more uniform caliber and they seem clearly to be continuous from sarcomere to sarcomere across the Z band Occasionally there is a partial reduplication of a triad resulting in a pentad consisting of 3 cisternae and 2 slender intermediate tubules

The finding of an unusually extensive and highly ordered sarcoplasmic reticulum in these 2 exceptionally fast acting skeletal muscles is consistent with the hypothesis[16] that this system of membrane bounded channels is involved in intracellular conduction of the impulse that activates the myofibrils

Sarcoplasmic Reticulum of Cardiac Muscle

Several considerations would lead one to expect that the sarcoplasmic reticulum might be less well developed in cardiac than in skeletal muscle Some of these are the smaller fiber diameter the central position of the nucleus which brings the contractile elements nearer to the surface the myogenic nature of the contraction the presence at frequent intervals along the cardiac muscle fibers, of specialized cell to cell junctions (intercalated discs) that may offer pathways of inward conduction from the sarcolemma not present

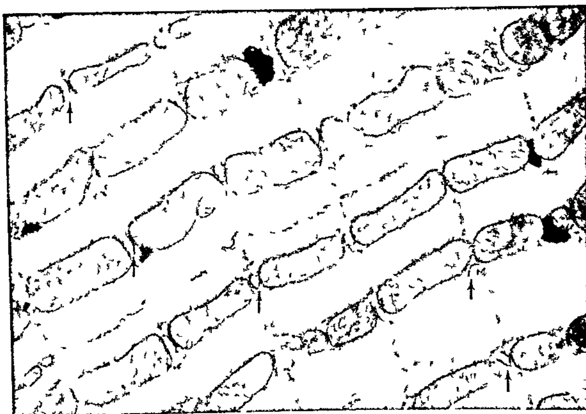


Figure 7

Another longitudinal section of the interior of a cardiac muscle fiber from the bat heart showing contracted myofibrils alternating with rows of mitochondria each about the length of one shortened sarcomere Again the arrows point out transverse elements of the reticulum located at the Z band instead of at the A I junction which is the usual location of the triads in mammalian skeletal muscle

in the syncytial fibers of skeletal muscle and finally the slower rate of contraction of heart muscle Nevertheless there is sufficient correlation between the rate of heart beat in various animal species and the degree of development of the sarcoplasmic reticulum to suggest that this system has a significant function in cardiac as well as in skeletal muscle In a previous study of the fine structure of the turtle atrium[17] the reticulum was found to be rudimentary Evidently it is not essential in the slow beating heart of this cold blooded species In the rat which has a rather rapid heart rate Porter and Palade[3] found a loose network of sarcotubes with relatively little differentiation in relation to the cross banded pattern of the myofibrils (fig 1B) Although small terminal cisternae were identifiable on either side of the Z band these did not form typical triads nor did they extend laterally for any considerable distance

We have recently studied the myocardium of the bat *Myotis lucifugus* These small mammals normally have a heart rate of the order of 500 to 600 per minute but under some physiologic conditions it may reach as high as 1 000 per minute Electron micrographs of longitudinal sections reveal an extraordinary number of large mitochondria of complex internal structure located in the clefts between myofibrils at the poles of the centrally placed nucleus and immediately beneath the sarcolemma

The mitochondria are often about the length

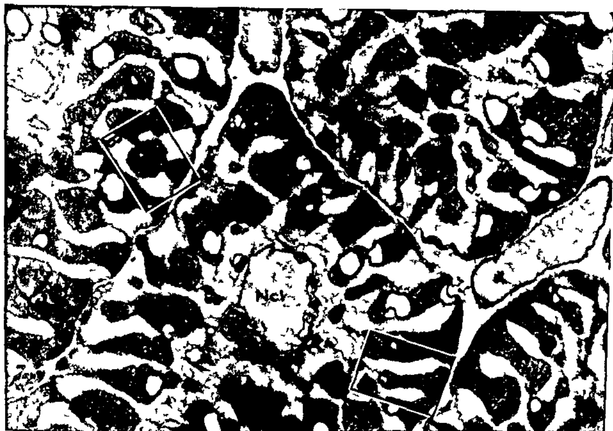


Figure 8

A low power electron micrograph of portions of 4 cardiac muscle fibers of the bat in transverse section. Observe the centrally placed nucleus (Ncl) and the fact that the large dense mitochondria occupy fully half the cross sectional area of the fiber. The clear areas that appear to be holes in the section are in fact lipid droplets whose content has been largely extracted during specimen preparation. The area enclosed in rectangle A is shown at higher magnification in figure 9 and that in rectangle B *constitutes figure 10.*

of a sarcomere. The periphery of a partially contracted fiber frequently shows a characteristic scalloped or corrugated appearance owing to the fact that the sarcolemma is closely adherent to each Z band of the outermost myofibrils but is separated from the myofibrils elsewhere by mitochondria. The mitochondria immediately beneath the sarcolemma would thus seem to be confined within relatively stable compartments bounded by the lines of adhesion of the sarcolemma to the Z band of successive sarcomeres (fig. 6). The structural basis for this close binding of the surface membrane to the Z band is not clear from the micrographs. Numerous lipid droplets are interspersed among the mitochondria that are more deeply situated in the fibers (fig. 6) and are evidently an important energy source in the rapidly beating hearts of this and other small mammals. Since bats hibernate, it would be of interest to know whether there are seasonal variations in the abundance of the myocardial lipid, but thus far our studies do not extend over a large enough span of time to throw any light on this subject.

Also located between the ends of the mitochondria at the level of the Z band are transversely oriented tubular elements of the sarcoplasmic reticulum, indicated by arrows on figures 6 and 7. These evidently correspond to the triads of the reticulum of skeletal muscle but tend to be single or at most double and

are placed at the Z band instead of in the A band near the A I junction One tends to underestimate the extent of the sarcoplasmic reticulum in cardiac muscle because of the peculiar geometry of its myofibrils They are not discrete fibrils uniformly round or polygonal in cross section as in skeletal muscle but instead exhibit a greater degree of confluence and branching so that in transverse sections the size of the myofibrils is variable and their shape highly irregular In consequence of the inconsistency of their tridimensional form and the prevalence of curving surfaces one rarely encounters such extended surface views of the sarcoplasmic reticulum in longitudinal sections as one sees overlying the more regular faces of the myofibrils in skeletal muscle It is necessary therefore to rely mainly on cross sections in attempting to construct a mental image of the 3 dimensional organization of the reticulum

In transverse sections of bat heart muscle viewed at low magnification (fig 8) one is struck by the irregular shape of the myofibrils around the centrally placed nucleus and by the great number of large mitochondria that take up nearly half of the cross sectional area of the fiber and occupy nearly all of the interfibrillar sarcoplasm In micrographs of higher magnification (figs 9 and 10) the mitochondria are found to conform very closely to the irregular contours of the surrounding myofibrils however in the narrow interstices between the two there are numerous circular profiles 400 to 500 Å in diameter (see at arrows) which are cross sections of the longitudinally oriented tubules of the sarcoplasmic reticulum Owing to the paucity of interfibrillar sarcoplasm these profiles are easily overlooked in low power micrographs but from the large numbers visible in micrographs of higher magnification it is clear that the sarcoplasmic reticulum is quite well developed in cardiac muscle of this animal species

To what extent is this tubular system developed in the human heart? Although there have been several brief reports on the fine structure of the human myocardium[18] none has devoted particular attention to the sarcoplasmic reticulum Our own studies on man are as yet too fragmentary to permit us to do more than to affirm its presence and to record some preliminary impressions on the degree of its development as compared to other animal species

In electron micrographs of human atrial muscle the myofibrils show the same orderly arrangement of two interdigitating sets of filaments that has been described in other striated muscles (fig 11) The myofibrils vary considerably in size and in cross sectional shape but are on the whole less pleomorphic than those described here for the bat The mitochondria which have a dense matrix and a complex internal membrane structure are numerous and are distributed singly or in sizeable clusters among the myofibrils The interfibrillar sarcoplasm is more abundant than in the bat myocardium The mitochondria being less crowded show less tendency to adopt unusual shapes conforming to the spaces between the myofibrils Among the mitochondria and in the clefts between adjacent myofibrils are tubular elements of a sparce sarcoplasmic reticulum (see arrows fig 11) Definite triads have not been identified at the level of the Z bands in our material nor have any clear connections been demonstrated between the loose meshes of the reticulum and the sarcolemma Although it is probably basically similar in its distribution and organization the reticulum in the human myocardium is evidently far less elaborately developed than is that of smaller mammals with a more rapid heart beat

Comment

The history of the discovery of the sarcoplasmic reticulum of striated muscle has been traced and the physiologic evidence for its participation in intracellular impulse conduction has been reviewed Our own comparative observations indicate that this system of membrane limited tubules is particularly well developed in certain exceptionally fast acting skeletal muscles These findings



Figures 9 and 10 *(See legend on opposite page)*

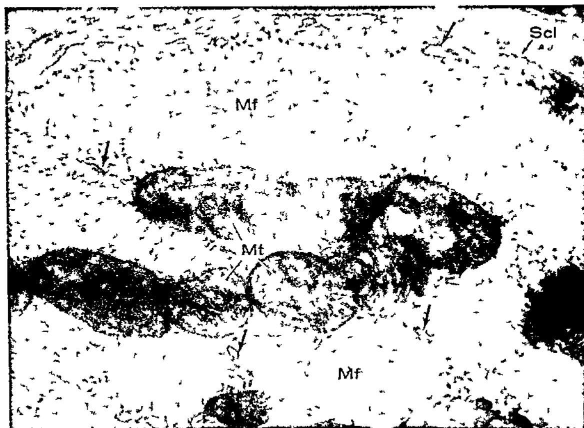


Figure 11

An electron micrograph of a small area at the periphery of a human cardiac muscle fiber The sarcolemma (Scl) is at the top of the figure and shows the usual coating of basement membrane material The mitochondria (Mt) are fairly large and rich in internal structure The myofibrils (Mf) show the usual precise hexagonal pattern of myofilaments The sarcoplasmic reticulum is less well developed than in the hearts of small mammals with rapid heart beats but several profiles of sarcotubules in cross section can be seen between the myofibrils in this figure (see at arrows)

are offered as further indirect evidence for the hypothesis that the reticulum is involved in the coupling of excitation to contraction It is recognized however that this canalicular system may function in other ways besides the conduction of an impulse by its limiting membrane It may prove to be important in the synthetic activities of the muscle cell or its lumen may provide a continuous pathway for distribution of energy rich compounds or other essential metabolites to the myofibrils

The sarcoplasmic reticulum has been shown to be less highly developed in cardiac than in skeletal muscle but it is so organized in

Figures 9 and 10

Higher magnification electron micrographs of two small areas of the cardiac muscle fiber shown in figure 8 The myofibrils (Mf) are highly irregular in shape and their surface closely conforms to the shape of the mitochondria (MT) which occupy nearly all of the interfibrillar sarcoplasm Between the myofibrils and the mitochondria are numerous small profiles of membrane bounded tubules in cross section (see at arrows) These are the longitudinal components of the sarcoplasmic reticulum

relation to the cross banded pattern of the myofibrils as to suggest that it may have a similar function in both The reticulum is rudimentary in the slow beating heart of the turtle but reaches a rather high degree of complexity in the very rapidly beating heart of the bat. The reticulum of the human myocardium has not been adequately studied but appears to be intermediate between these extremes It is not possible now to state how important a role the sarcoplasmic reticulum plays in the physiology of the human heart but new findings in research often turn out to have far more significance than at first seems likely It may not be too fanciful to imagine that a few decades hence the cardiologist may be concerned with functional disturbances of this intracellular communication system just as he is concerned today with defects of conduction at a grosser tissue level

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The Structure of the Specialized Impulse-Conducting System of the Steer Heart

By JOHANNES A G RHODIN M D PETER DEL MISSIER M D AND L CORSAN REID M D

The specialized impul e originating and conducting system of the steer heart has been analyzed with the light phase contrast and electron microscopes after careful dissection of its gro s anatomic parts The specific tissue is compo ed of cells with distinct cell boundaries No syncytium exists The cells of the sino atrial (S A) and atrioventricular (A V) nodes closely resemble those of the common myocardium They conne t end to end via intercalated disks most of which represent the starting point and termination of numerous myofibrils The cells of the bundle of His and its distal branches are large and spindle shaped and joined in a staggered fa hion They display a fair number of myofibrils The cell contact is established by numerous desmosome that only rarely connect with myofibrils It is believed that the multiple desmosome type of connection p esent in most parts of the specific tissue of the steer heart indicates that th s t ssue has maintained its embryologic appearance to a large extent The rol of the desmosomes in facilitating the propagation of the impulse throughout the specific t ssue is discus ed

IT SEEMS APPROPRIATE to discuss the structure of the specialized conducting system of the heart at a symposium on the myocardium Without this tissue the stimulus for the contraction of the common myocardium would hardly ever be originated Thus it is to be expected that certain unique peculiarities will characterize the cells of the specialized conducting system An account of their ultrastructure will be given here in the hope that this will explain among other features of this tissue its ability to transmit the impulse of contraction so much faster than the cells of the common myocardium

Although the most fundamental mechanism of this system is the origination of an impulse there has been a long lasting controversy regarding its existence as a specific tissue in the heart [1] This is due principally to its great variation in style and structure throughout the animal kingdom including mammals At present this controversy is settled and the existence of a specific tissue in man [3, 4] sheep [5, 6] and steer[7] is structurally and functionally beyond any doubt In the reader s own mind however it may not be quite clear whether or not the specific tissue is muscular or nervous Here new technics such as phase contrast and electron microscopy have been most helpful in demonstrating that the so-called conducting system is a true contractile muscular tissue

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Material and Methods

The material used in this study has been exclusively the specific tissue of the steer heart The hearts were obtained and dissected in the slaughter house within 15 minutes of the animal being killed Osmium tetroxide was used as a fixative[8, 9, 10] and liquid plastic as an embedding medium[11] for specimens prepared for electron microscopy Thick sections for phase contrast microscopy and thin sections for electron microscopy were cut with the LKB Ultrotome [12] The Siemens Elmiskop I electron microscope was employed

Results

Anatomy of the Conducting System

The S A node is regarded as the pacemaker often called the node of Keith Flack. In man as well as in the steer there is no direct continuity via specific tissue between the cells of the S A node and the A V node of which the latter forms the beginning of the ventricular part of the conducting system In the

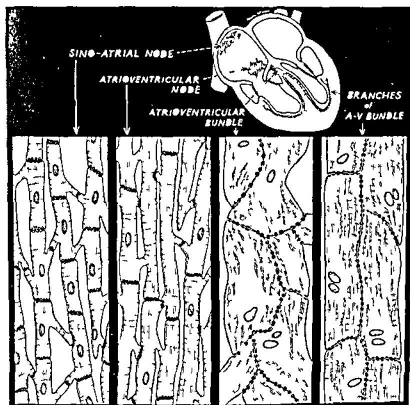


Figure 1

A schematic representation of the gross anatomic histologic and to some extent the ultra structural organization of the conducting system of the steer heart The four areas represent camera lucida drawings at a magnification of about 150 times The size of the intercalated disks and the desmosomes is somewhat exaggerated in order to facilitate a comparison of their ultra structure

steer[7] the A V node or node of Tawara is quite large and diamond shaped (fig 1) The A V node is followed directly by the A V bundle also called the common bundle or the bundle of His This structure eventually splits up into a left and a right branch each of which in turn gives rise to a network of fine ramifications that terminate in direct contact with the myocardial fibers

Structure of the Common Bundle

Light Microscopy

The structure of the common bundle will be considered first for in the present material the cells that compose this part of the conducting system are easily recognized because of their pronounced difference in shape and size from those of the common myocardium justifying the term specific tissue This difference is easily seen even with the light microscope (fig 2) Here the size of the specific tissue cells can be compared with that of the common myocardium It is obvious that the cross section diameter of the specific cells is at least twice that of the cells of the common myocardium The common bundle is rich in connective tissue elements No true inclosing of connective tissue can be identified as in the sheep heart The individual fibers of the common bundle are however sharply delimited from the surrounding connective tissue as seen in cross section in the phase contrast microscope (fig 3) The interior of the fiber is divided by delicate lines that give rise to irregularly shaped areas In a longitudinal section of a similar fiber (fig 4) the lines and areas can be recognized Nuclei are present in several areas or fields In addition there are longitudinally arranged fibrils that bear a well defined cross striation In other words we are dealing here with a conducting fiber that is composed of a number of cells put together not only end to end but also side to side in a staggered position because of their obvious spindle or multiangular shape Because of the cross-striated appearance of the cellular fibrils it is evident that the cells represent muscle cells

Electron Microscopy

The cells of the conducting system fibers are about 100 to 200 microns long in the common bundle In the coarser fibers 5 to 7 cells may be seen in a cross section (fig 3) but finer fibers with only two cells also occur (fig 5) The fiber is surrounded by a thin basement membrane with a varying number of reticular and collagenous fibrils The basement membrane does not penetrate between the individual cells that build up the fiber The boundaries of cells are difficult to see in the low magnification electron micrographs (figs 5 and 6) but their course can easily be traced because of the relatively heavy accumulation of cross-striated myofibrils along their surfaces Indirectly the various cell territories can be distinguished also by the variation in density of the cytoplasm in different cells (fig 6)

The cells of the common bundle have few cytoplasmic organelles as compared with the cells of the common myocardium More than 50 per cent is occupied by what the early cytologist would have called ground cytoplasm In the electron micrograph this part of the cytoplasm is characterized by a fine granularity with the granules having an average diameter of about 200 A (fig 7) This is close to the diameter of the submicroscopic granules that are abundant in the exocrine cells of the pancreas where they have been demonstrated to contain ribonucleoprotein [13] However histochemical techniques have shown that the light microscopically clear areas of the specific tissue cells do contain glycogen [6] The fine granularity is therefore believed to represent glycogen similar to that which has been demonstrated in the myocardium of the turtle atrium [14]

Another submicroscopic component is represented by small vesicles of varying size The vesicles have a clear center and are bordered by a smooth membrane (fig 7) They do not seem to be derived from the cell membrane and their function is unknown They may be abundant in one cell and completely absent in another (fig 6) which possibly indicates variation in function



Figure 2

Light micrograph of the common bundle (C) and the myocardium (Y) The cells of the common bundle are large and pale whereas those of the myocardium are small and dense (Formalin fixation hematoxylin eosin stain × 250)

Other cell components that can be seen with the aid of the light microscope are nuclei mitochondria myofibrils and occasional lipid droplets There is more than one nucleus to each cell The nuclei usually occur in pairs and are located in the center of the cells of the common bundle A heavily stained nucleolus which has a loose structure is always present (fig 6)

The *mitochondria* are small and spherical They display the common fine structural pattern of a thin triple layered outer membrane and several triple layered inner membranes (fig 7) The matrix of each mitochondrion is quite loose in the present material This may indicate less good preservation than desired and could depend on the relatively long lapse of time between the killing of the steer and the moment when

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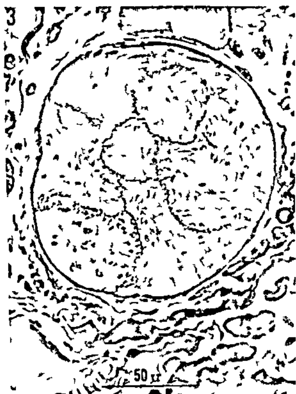


Figure 3

Phase contrast micrograph of a cross sectioned fiber of the common bundle The fiber is sharply delimited toward the surrounding connective tis sue The lines in the interior of the fiber represent cell borders (Osmium tetroxide fixation, unstain ed plastic ection × 500)

these delicate and sensitive organelles were impregnated by the slowly penetrating os mium tetroxide The mitochondria are usu ally *found along the cell borders and also* closely associated with the myofibrils

The most prominent components of the cells of the common myocardium are the *myofibrils* They vary greatly in length and width (figs 6 and 7) Most are freely dis persed in the cytoplasm but some begin and terminate at the cell membrane here in close association with the so-called desmosomes This arrangement is however much more pronounced in the distal branches of the conducting system Only rarely have struc tures been identified that resemble the Purkinje filaments demonstrated by Muir in the sheep heart [6]

Each myofibril shows the fine structure (fig 7) that has been described in both the skeletal[15, 16, 17] and the heart muscle [14, 18] Myo*filaments are the main components* with the traditional thickenings associated with the various bands (see Huxley in this sympo sium) The Z bands are of particular interest in the myofibrils of the specific tissue cells In the cells of the common myocardium and the skeletal muscle this structure is known to be composed of split myofilaments embedded in an amorphous electron dense substance In the present material it has been found that every now and then the dense amorphous substance extends beyond the territory of the myofibril and establishes a direct continu ation with a similar dense amorphous band that in some instances accompanies the cell borders at a distance (fig 7) In order to understand this relationship fully it will be necessary to develop the concept of an inter cellular relationship in the common bundle

The *cell border* follows an irregular and wavy course (figs 7 and 8) The plasma membrane of each cell is quite delicate and seems to be thinner than recorded for cells elsewhere in the body [19] Two structures are associated with it One is represented by the just mentioned continuous electron dense band, which roughly follows the course of the cell border, although some distance re moved The second structure is closely as sociated with the plasma membrane and is attached for short interrupted areas at its intracellular aspect The plasma membrane of either specific tissue cell is about 50 Å thick, and the intercellular space charac terized by less electron density measures about the same As the dense structures asso ciated with the intracellular aspect of either plasma membrane are approached the inter cellular space widens to about 175 Å and becomes occupied by a dense substance in which possibly cross striations may be dis tinguished At higher magnification another membranous layer can be resolved which is embedded in the cytoplasmic dense zone (fig 9) This second layer is identical in size with the

plasma membrane and also parallel to it The entire structural complex has been analyzed and described previously in the ventricles of frog mouse and guinea pig hearts[17 0-] in the frog heart and papillary muscle of the dog heart [3] and in the turtle atrium[14] Sjostrand and co workers[1 0] refer to this structure as the S region, whereas the school of Fawcett[14] prefers the more commonly accepted term desmosome We should like to use the term desmosome for this structure in the common bundle of the steer heart because it closely resembles the desmosomes seen in a variety of epithelial cells most advantageously in the epidermis [4 6] If we now return to the *continuous dense band,* it becomes evident that this structure is a cytoplasmic condensation similar in appearance to the desmosomes It is obvious that in the present material the dense band is not applied to the plasma membrane but is parallel to it and that it can be seen to connect desmosomes with each other Every so often a connection is also established with the Z band of a nearby myofibril In concluding it should be stressed that the cells of the specific tissue as analyzed in the common bundle of the steer heart are provided with desmosomes the great number of which was not previously known The functional interpretation of this arrangement will be discussed after we have considered the cellular contacts as they appear in the S A and A V nodes

Structure of the Distal Branches of the Common Bundle

As the distal branches of the A V bundle are approached the cells become longer They meet preferably end to end in addition to their side to side contact (fig 10) The number of myofibrils increases considerably and they are definitely longer and more parallel in arrangement when compared to the cells of the common bundle The increased number of myofibrils gives less space for accumulation of glycogen The nuclei also appear in pairs here mostly located toward one end of the cell and close to the lateral cell border

The fine structure of these cells is identical with that of the common bundle cells Among other things this implies that the multiple desmosome type of connection is maintained The dense band with a course parallel to the plasma membrane is seen less often



Figure 4

Phase contrast micrograph of a longitudinally sectioned fiber of the common bundle The spindle shaped cells are arranged in a staggered fashion A number of cross striated myofibrils are present (Osmium tetroxide fixation unstained plastic section × 500)

Structure of the S A and A V Nodes

Light Microscopy

In the steer heart earlier investigators have demonstrated by light microscopy that the cells of both nodes resemble those of the common myocardium Grossly one can tell them apart because the nodal fibers are surrounded by an abundance of connective tissue elements and by the comparatively loose arrangement of individual fibers (fig 11) These circumstances have been used as criteria in the present study

In a cross section of S A fibers (fig 12)



Figure 5

Low magnification electron micrograph of a cross sectioned thin fiber of the common bundle The entire width is occupied by 2 cells (C1 C2) The course of the two cell membranes facing each other is indicated by the arrows Most of the irregular dense spots represent cross sectioned myofibrils A basement membrane (BM) surround the entire fiber uninterruptedly (× ~ 700)

is seen with the phase-contrast microscope the individual cells are fairly loosely arranged and the cross sectioned myofibrils in the interior of the cells are quite scarce In a cross section of A V nodal fibers (fig 14) using the same magnification the cells are somewhat more closely arranged and show a greater abundance of myofibrils In longitudinal sec

tion the S A node (fig 4) s similarity to the struc ium of the at municate by s work known issue itself to end in nt

ows a striking the common individual s giving



Figure 6

Low magnification electron micrograph of a longitudinally sectioned fiber of the common bundle. At least 4 cells (C1–C4) are seen. The overall density of the cells varies because of a variation in the finely granulated cytoplasm. The cell C4 is most dense and contains a great number of submicroscopic vesicles of unknown origin and function. The myofibrils are widely scattered but tend to become aggregated along the cell borders. Most fibers are thin (F1) but occasional thick ones (F2) occur. The nucleus (N) has a heavily stained but loosely arranged nucleolus. (× 2,[illegible]00.)

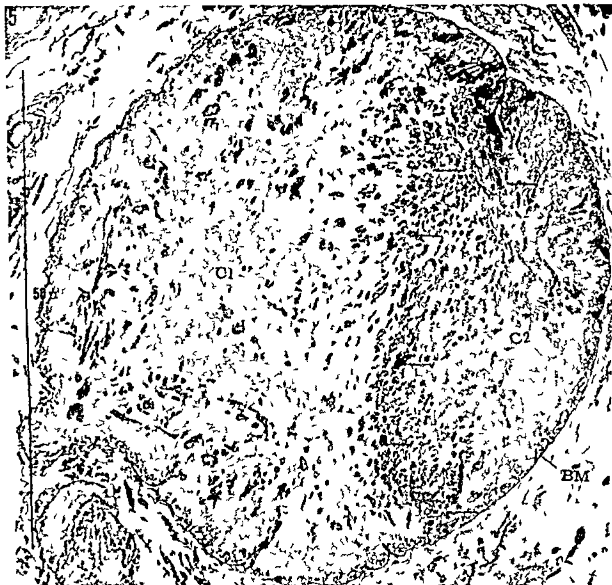


Figure 5

Low magnification electron micrograph of a cross sectioned thin fiber of the common bundle The entire width is occupied by 2 cells (C1 C2) The course of the two cell [membranes] facing each other is indicated by the arrows Most of the irregular dense spots [represe]nt cross sectioned myofibrils A basement membrane (BM) surrounds the entire [fiber] uninterruptedly (× 2 500)

as seen with the phase contrast microscope the individual cells are fairly loosely arranged and the cross-sectioned myofibrils in the interior of the cell are quite scarce In a cross section of A V nodal fibers (fig 14) using the same magnification the cells are somewhat more closely arranged and show a greater abundance of myofibrils In longitudinal section the S A node (fig 13) shows a striking similarity to the structure of the common myocardium of the atria The individual fibers communicate by side branches giving rise to a meshwork known to exist in the cardiac muscle tissue itself The nodal cells usually meet end to end marked by the dense cross lines that represent intercalated disks



Figure 8

The junction of 3 common bundle cells (C1 C3) In C1 a l ng and den e cyto plasmic band can be traced whereas this structure is not present in C2 and C3 On the other hand s veral desmosomes (arrows) of varying length can be seen here The rec tangle is enlarged in figure 9A (× 18,500)

similar to the structures of the common myo cardium A relatively large number of nuclei and disks are present indicating that the nodal cells are comparatively short It is easy to obtain a section of the S A node where the fibers are parallel but this is not so in the A V node (fig 15) Here the fibers tend to run in many directions a fact demonstrated by earlier investigators The nodal cells here are joined end to end as in the S A node but they are longer judging by the relatively fewer intercalated disks and nuclei

Apart from this the A V nodal fibers are thicker and more densely arranged and seem to contain a larger number of myofibrils than do the cells of the S A node

Electron Microscopy

At the ultrastructural level there is basi cally little difference between the cells of the S A and the A V nodes in the steer heart Therefore only the fine structure of the cells of the S A node will be considered

The organization of the cells of the S A node is very much the same as that of the

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larity is believed to represent gl jcogen The myofibrils (F) displa j thin filaments that have the usual characteristics of myofilaments The Z bands (Z) are most prominent the H bands (H) with the central thin M disk can also be seen This particular myo fibril is contracted because the light I bands on either side of the Z band cannot be observed The dense cytoplasmic band that accompanies the cell borders is oc asionally seen ( ) to make a connection with a Z band (× ~3 100)

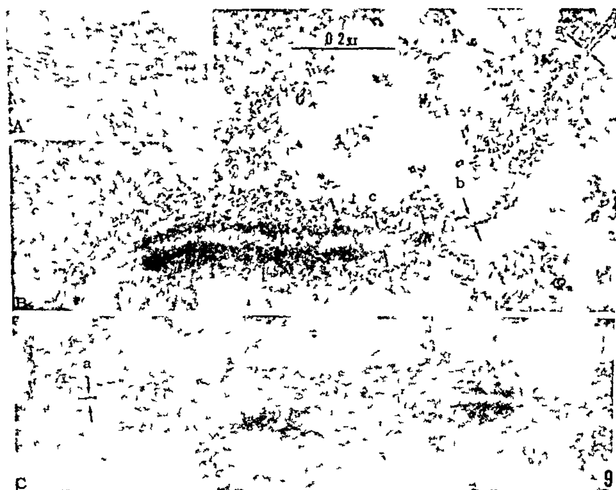


Figure 9

Detail of the desmosomes present in the cells of the common bundle They vary considerably in length The plasma membranes are about 50 Å thick and separated by a 0 Å wide intercellular space (a) Closer to the desmosome (b) the intercellular space widens and reaches a width of about 175 Å within the desmosome (c) A second dense layer parallel to the plasma membrane is resolved within the desmosome (most clearly seen in A and C) (× 128 000)

common myocardium (fig 16) Indeed the similarity is so striking that it would be virtually impossible to tell them apart if reference could not be made to the anatomy and the histology of the tissue then the two tissues look different according to earlier investigators and also to our own description here Because of the similarity between the cells of the S A node and the common myocardium we do not find it necessary to go into a lengthy discussion about their ultrastructure but refer the reader to earlier electron microscopic investigations of heart muscle [14 18 20 3 5] However a few points of importance in reference to the present problem will be brought up

Each cell is enclosed by its plasma membrane a delicate envelope about 70 Å thick The cells vary in length and meet only end to end with the point of contact marked by the intercalated disk Individual cells branch and interconnect with neighboring cells to form a three dimensional meshwork of muscle fibers The cells are also wrapped by a thin basement membrane that does not penetrate between the cells (fig 17) Usually each cell has 1



Figure 10

Phase contrast micrograph of a longitudinally sectioned fiber of the left distal branch of the common bundle The cells are more elongated than in the common bundle and they meet in preference end to end (arrows) They contain a fairly large number of cross striated myofibrils (Osmium tetroxide fixation unstained plastic section × 500)



Figure 11

Light micrograph of the sino atrial node (SA) and the myocardium (Y) The size of the cells is approximately identical in both instances but the cells are more loosely arranged in the S A node (Formalin fixation hematoxylin eosin stain × 100)

nucleus but occasionally 2 nuclei can be seen in the same cell Seen in a section about 75 per cent of the cell is occupied by myofibrils characterized by the well known pattern of cross striations Mitochondria are distributed along the plasma membrane and in between the myofibrils

The *intercalated disk* runs across the entire width of the nodal fibers (fig 18) For many years it was believed that the common myocardium consisted of cells without true cell borders thus forming a syncytium With the aid of the electron microscope [14 18 20 3 9] however it became clear that the intercalated disk represents the junction of cardiac cell a fact that once and for all overthrew the concept of myocardial syncytium [9 30] The cells of the nodal fibers in the steer heart are joined similarly As the myofibrils approach the intercalated disk the individual myofilaments seem to spread slightly before they attach at the disk (fig 18) The intercalated disk is actually composed of short dense condensations or subunits of the cytoplasm adjoining the intracellular aspect of the plasma membrane of the ends of two neighboring cells The myofilaments that approach the end of the nodal cell then establish an anchorage in the dense subunits at the plasma membrane (fig 19) The plasma membrane is always rather wavy at the intercalated disk In between the dense subunits it appears smooth and devoid of attached cytoplasmic structures Occasionally however the desmosome type of cell to cell contact can be identified



Figures 12 and 13

Phase contrast micrographs of cross sectioned (fig 12) and longitudinally sectioned (fig 13) fibers of the S A node of the steer heart The fibers are loosely arranged with a fair number of myofibrils Some of the intercalated disks are indicated by arrows (Osmium tetroxide fixation unstained plastic sections × 500)

but these structures do not receive myofibrils This structure has been found and carefully analyzed in the common myocardium of mouse and guinea pig hearts by Sjostrand and co workers It was called an S region and corresponds structurally to the desmosomes described here in the cells of the common bundle of the steer heart

Discussion

Anatomy

At present there is no doubt that a specific tissue does exist in the myocardium of the steer heart The dissections by del Missier et al [7] demonstrated that it is fairly easy to show its gross anatomic distribution not only in the heart of the steer but also in those of man dogs pigs and sheep The histologic appearance of the specific tissue was most convincingly analyzed by Blair and Davies [1] in the bovine heart Our light and phase contrast microscopic studies essentially confirm those of Blair and Davies The remarkable similarity between the structure of the common myocardium and the S A and A V nodes should again be stressed The only gross difference seems to be the comparatively loose arrangement of the fibers of the nodes with the interspaces occupied by connective tissue elements

Electron Microscopy

Earlier investigations of the specific tissue by means of electron microscopy have dealt with this structure in the myocardium of the sheep heart Muir [6] who made the first electron microscopic study of the specific tissue analyzed the distal branches of the common





Figures 14 and 15

Phase contrast micrographs of cross sectioned (fig 14) and longitudinally sectioned (fig 15) fibers of the A V node of the steer heart The fibers are arranged more densely in the A V node than in the S A node and they also contain a greater number of myofibrils The cells are longer in the A V node and therefore display a smaller number of intercalated disks (arrows) (Osmium tetroxide fixation unstained plastic sections × 500)

bundle whereas Caesar et al concerned themselves with the bundle of His and the false tendons These works give an accurate account of the fine structure of the specific tissue cells in the sheep heart which for the most part conforms with the present analysis of the steer heart The work by Muir[6] in particular is most elucidating It demonstrates clearly the difference between the cells of the common myocardium and those of the specific tissue and in our opinion describes the cell to cell relationship more clearly than does that of Caesar et al

However there seem to be certain differences at the ultrastructural level between the cells of the sheep and of steer specific tissue These may be of minor importance in relation to the function of this tissue but are sufficiently pronounced to justify a short discussion of their significance The scattered myofilaments that Muir[6] found and called Purkinje fibrils do not seem to occur in our material Loosely arranged myofibrils do occur but without exception they display the normal bandings that the Purkinje fibrils of the sheep seem to lack The significance of these findings cannot be pointed out until the specific tissues of humans and several other animals have been investigated

Another feature is the occurrence of submicroscopic granules most of which Muir[6] and also Caesar et al[5] interpret as representing ribonucleoprotein particles similar in nature to those demonstrated in the exocrine



Figure 16 *(See legend on opposite page)*

Figure 17

Cross section of 3 fibers (C1–C3) of the S-A node. The larger of the cells is sectioned through the nucleus (N). It is obvious that at least two thirds of the fiber area is occupied by myofibrils. (× [illegible],000.)

Figure 16

Low magnification electron micrograph of longitudinally sectioned fibers of the S-A node. The central muscle fiber has nuclei (N) and is like the neighboring ones enveloped by a basement membrane which gives the surface of the fiber a scalloped appearance. Although the fibers sometimes are closely apposed, there is always an interstitial space (arrows) in which capillaries (C) are also seen. The myofibrils with their cross striations are numerous. The intercalated disks (★—★) represent true intercellular contacts without intervening basement membrane. (× [illegible]100.)

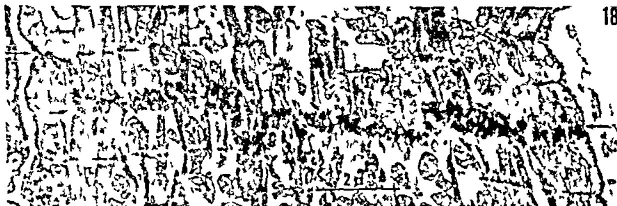


Figure 18

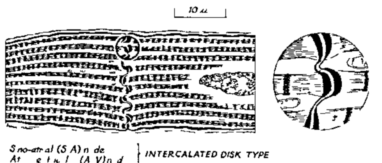
Intercalated disk area of a longitudinally sectioned fiber of the S-A node. The disk runs across the entire width of the fiber (arrows). The myofilaments of each myofibril (F) seem to become more loosely arranged before they anchor at the dense structure of the intercalated disk. (× 10,500.)

cells of the pancreas by Palade.[31] In our material we cannot confirm that the outer component of the nuclear membrane is studded with these granules, neither can we find vesicles provided with these submicroscopic particles. On the contrary, we find these particular membranes to be smooth and conclude that neither structure represents part of a rough-surfaced endoplasmic reticulum.[3] The vesiculation at the intercalated disks and the large vacuoles which supposedly are derived from retraction of the plasma membranes as suggested by Caesar et al. have not been recorded in the steer heart. This may be a species difference but could also be explained by poor fixation of the material analyzed by Caesar et al. Again the ultimate solution to this problem has to await further analyses of this tissue in other animals.

Functional Considerations

From a functional point of view, it seems of greatest importance to compare the two different types of cellular attachments that are encountered in the various parts of the conducting system of the steer heart. In the S-A and A-V nodes the intercalated disk type of the common myocardium prevails although the desmosome type of structure may also be found to a lesser degree in between the dense subunits of the intercalated disk. In the A-V bundle and its left and right branches including their fine ramifications, the multiple desmosome type of attachment has been found exclusively in the present study of the specific tissue of the steer heart (fig. 19).

The function of these two types of structure is obviously to secure the mutual attachment of cells, a conclusion reached by comparison with other instances in which an abundance of desmosomes undoubtedly serves this purpose, e.g., in the epidermis.[4, 6] Ontogenetically, it is of interest to recall that Muir[8] has demonstrated by electron microscopy that the multiple desmosome type is present in the common myocardium of the rabbit in embryos from 18 days after coitus to birth. Later on, the adult type is developed with the characteristic wavy course of the cell boundary within the intercalated disk. This seems to indicate that in the steer heart the cells of the common bundle and its branches with the multiple desmosome type may represent a tissue that has maintained its embryologic appearance. The specific tissue may therefore represent the vestigial remnant of the first vascular tube; phylogenetically it would be the oldest tissue in the heart and ontogenetically the first to appear. There may also be another function pertaining to the multiple desmosomes versus the intercalated disks. Sjostrand and co-



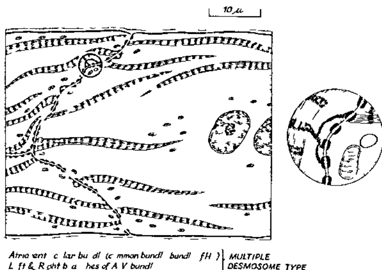


Figure 19

Schematic representation of the cellular contacts as seen in the various parts of the impulse conducting system of the steer heart

workers[9] have suggested that the specialized regions they found in the common myocardium between the disk regions and that closely resemble our desmosomes may represent areas with a lower ohmic resistance and may form paths with a great safety factor for conduction across the cell junction. Our findings of a multitude of desmosomes in the lower regions of the conducting system where certainly the impulse travels so much faster than in the common myocardium seem to support this hypothesis.

Finally, we may, in summing up, compare the histologic and ultrastructural appearance of the various parts of the conducting system? From our studies it is fairly clear that the passage of the stimulus in any part of the conducting system could occur by a wave of muscle contraction. Any electrical effects produced by this wave of contraction are undoubtedly the results of previously activated enzymatically catalyzed chemical processes. Bourne[33] has reported the presence of alkaline phosphatase in the intercalated disks of the common myocardium and this enzyme may well be present in the disks of the specific tissue also. Succinic dehydrogenase[33, 34] and cholinesterase[3] are both concentrated along the borders of the specific tissue cells, presumably in the mitochondria but possibly also located in the small vesicles described here. If the transmission of the impulse is facilitated by the intercalated disks and the desmosomes and if the impulse is mediated by enzymes located in or near these structures, then one would expect it to travel faster across a border where these structures are frequent. We do not know the rate of conduction in the S A node itself but it is 1 000 mm per second in the atrial muscle and the number of intercalated disks per 40 000 square microns in a longitudinal

section of the atrial muscle is about equal to that of the S A node (fig 13) possibly indicating that the rate of conduction is roughly the same The rate of conduction in the A V node is about 200 mm per second and in the ventricular muscle 300 to 500 mm per second Again there is a close structural resemblance between the A V node and the ventricular muscle Furthermore the slower rate as compared with the atrium may be explained by the smaller number of intercalated disks and the larger size of the cells Once the impulse reaches the tissue of the common bundle the rate of conduction increases to 3 000 to 5 000 mm per second possibly explained by the great number of desmosomes and the large area they represent per square *unit of cell surface*

A purely mechanical point of view could also be introduced at this moment The structure of the S A node the A V node and the common myocardium of the atria and ventricles closely resembles that of a fisher man s net with a third dimension added whereas the structure of the common bundle and its branches reminds one of a set of strings connected here and there along its course (fig 1) A pull at one corner of a net would spread in all the directions of the net s meshes thus delaying the pull to be felt at the opposite corner of the net A pull at one end of a set of strings would be felt much faster at the opposite end be cause no spreading in a wide three dimen sional meshwork is involved

Of course many other factors would have to be taken into consideration[38] in explaining the high rate of conductivity of the cells of the specific tissue as well as in explaining the magic ability of the entire conducting system to originate autonomously a stimulus for contraction Here such factors as the high content of glycogen[1] as well as of sphingomyelin[37] probably play an important role

Acknowledgment

We are indebted to Dr A A Angrist for help and advice in dissecting the hearts We also grate fully acknowledge the skillful technical assistance of Miss Inger Alexanders on and the excellent artistic work by Miss Mary Lorene

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Blood Capillaries of the Heart and Other Organs

By George E. Palade, M.D.

The article is a review of work recently carried out on blood capillaries by the author in collaboration with Drs. M. G. Farquhar, G. Majno and S. I. Wissig.

It reviews the morphology of these vessels at the electron microscope level and confirms the existence of at least 3 distinct types of blood capillaries in small laboratory mammals. It shows that the capillary wall consists of 3 concentric layers (endothelium, basement membrane and adventitia) and indicates that the basement membrane forms a continuous layer in all capillaries so far studied.

Experiments in which colloidal gold particles were used as a tracer have shown that in capillaries with a continuous endothelium (muscle capillaries) the particles are transported across it by "pinocytic" vesicles. At the end of this step they must still transverse the basement membrane.

Experiments on glomerular capillaries, which typically have a discontinuous endothelium, were carried out on normal and nephrotic rats using ferritin as a tracer. By its accumulation on the luminal side of the basement membrane, the ferritin has identified this layer as the main filtration barrier.

A similar function of the basement membrane was demonstrated in muscle venules and venous capillaries by experiments in which the endothelium was rendered discontinuous by local treatment with histamine and serotonin.

THERE ARE, I believe, a good number of reasons for a renewed interest in problems of capillary permeability. To begin with, it is clear that we are dealing with a basic process in the physiology of metazoa. In these complex organisms the life of the multitude of cells in the intimacy of tissue depends in ultimate analysis on the ample and continuous exchanges that take place across the wall of capillary vessels between the blood plasma on one side and the interstitial fluid on the other. To continue, many pathologic conditions can be traced back to circulatory disturbances in general, and to variations in capillary permeability in particular, an outstanding example being the inflammatory process. And to finish, the mechanisms involved in the exchange of large quantities of water and solutes across the capillary wall are still poorly understood.

Studies on the structural aspects of the problem carried out over many years by light microscopy have revealed only a few general morphologic features that facilitate the exchanges: one could list under this heading the small diameter of the vessels, their organization into a tight meshwork that ensures a high surface to volume ratio for the circulating blood, and finally the extreme tenuity of the capillary wall. A description of these features together with an attempt to quantitate them in terms of capillary volume and capillary surface per unit volume in various tissues figured prominently—for instance—in August Krogh's book "The Anatomy and Physiology of Capillaries," which summarized what was known in 1928 about the morphology and physiology of blood capillaries.[1] In this book, which had considerable influence on the subsequent development of the field, Krogh assumed that the capillary wall consisted only of a layer of endothelial cells* but, aside from stressing their extreme thinness, did not further inquire into structural devices directly

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*The existence of a second layer, called basement membrane ("Grundhäutchen"), was described, however, by many histologists. See for instance Benninghoff.[2]

involved in capillary permeability With this in mind it would be fair to say that we inherited from our light microscope predecessors a good knowledge of the general layout of the capillary vessels but practically no knowledge regarding their fine structure and especially the structural details involved in capillary permeability

By comparison the physiologic aspects of the problem have been more thoroughly investigated and seem to be better understood Ever since Starling s 1896 hypothesis[3] it has been assumed that the force that drives the fluid out of or into the capillary vessels is the difference between the hydrostatic and osmotic pressures of the blood plasma † More recently however it has been realized that exchanges that operate on this basis are rather modest (a few per cent of the total) and that the mechanism in question may be more important for maintaining the blood volume than for carrying through adequate exchanges between the blood and the tissues In this respect it is generally agreed at present that diffusion plays the major role but difficulties are encountered when physiologic results are interpreted in structural terms Pappenheimer and his collaborators[4] for instance conclude that the permeability characteristics of the capillary wall could be explained by assuming that the wall is a rigid partition provided with patent permanent pores of ~ 60 Å effective diameter whereas Chinard and his colleagues[6] believe that the wall behaves like a laminar gel permeated by a continuous aqueous phase—fibrils and interfibrillar spaces in the gel being of molecular dimensions

It should be pointed out that in all these structural extrapolations it is assumed that the capillary wall consists of a single cellular layer the endothelium In Pappenheimer s formulation the postulated pores occupy a small part of the wall surface (~ 0 1 per cent) and are presumably located along the cell junctions cutting—so to say—through the intercellular cement a hypothetical substance that fills all the intercellular spaces of the endothelium The hypothesis according to which the exchanges between blood and tissues are carried through these cement filled spaces was originally advanced by Chambers and Zweifach[7] Before ending this short review of the physiologic aspects of the problem I should add that the capillary wall is more permeable to lipid than to water soluble substances For this reason Pappenheimer[4] and Renkin and Pappenheimer have actually postulated a double pathway across the wall via the pores for water and solutes and via the cells proper for lipid soluble substances

The Fine Structure of Blood Capillaries

This was the general state of our knowledge before the electron microscope was used to investigate the structural aspects of the problem The reinvestigation has already provided a large body of information and has established firmly at least two points [7, 8, 9]

First The capillary wall is a multilayered structure In addition to a cellular endothelial layer which could be described as an internal tunic it comprises an acellular layer —the basement membrane (middle tunic)— and an outer discontinuous stratum of cells and fibers that constitute an adventitial tunic

Second Although similar in their general construction capillary vessels differ constantly and characteristically in their structural details from tissue to tissue or rather from groups of tissues to groups of tissues The differences affect primarily the cellular layers the endothelial and the adventitial which could be discontinuous or even absent whereas the basement membrane generally persists as an uninterrupted layer This continuity of the middle tunic or basement membrane emerges then as a common structural feature for practically all types of capillary vessels so far examined *

†Actually the algebraic sum of the following terms hydrostatic pressure of the blood plasma osmotic pressure of same hydrostatic pressure of interstitial fluid (tissue pressure) and osmotic pressure of same

* See however Bennett et al and Wood[10] on the problem of hepatic blood sinuses

With this preparation we can start reviewing the electron microscopic evidence.* Figure 2 illustrates the type of capillary encountered in a skeletal muscle and in other tissues of the soma but also present in certain viscera, e.g., the myocardium and the smooth muscle of the digestive and reproductive tract. It is characterized by a continuous endothelium 0.5 to 0.2 μ thick and a continuous basement membrane. The endothelial cells are extremely flat but otherwise similar in organization to other animal cells. They possess a nucleus, a centrosphere region with 2 centrioles and a few small piles of smooth surfaced cisternae, a more or less developed endoplasmic reticulum, ribonucleoprotein (RNP) particles, mitochondria, and a cytoplasmic matrix in which fine fibrils can sometimes be detected. The only distinguishing although not unique feature is represented by a large number of vesicles[11, 1] located immediately below the cell membrane along both the blood and the tissue fronts of the cell (fig. 1). A closer examination reveals that some of these vesicles are open to the cell surface while others are closed. Those open could be described as invaginations of the cell membrane since their limiting membrane is continuous with the cell membrane. In this situation their content is also continuous with the extracellular fluid, blood plasma on one front of the cell and interstitial fluid on the other. Between closed and open vesicles a whole spectrum of possible intermediates is encountered. Grazing sections show the vesicles are quite numerous—120 to 140 per μ^2—on the luminal as well as on the

*The morphology of blood capillaries was studied in a number of small mammals (rats, hamsters, guinea pigs, rabbits) by electron microscopy using tissue specimens fixed in OsO_4 (buffered at pH 7.4 to 7.6) and embedded in methacrylate.

The experimental work was carried out exclusively on rat using the same preparative procedures for electron microscopy.

Explanation of Plates

All figures represent electron micrographs of rat blood capillaries. The corresponding tissues were fixed in osmium tetroxide and embedded in methacrylate. The sections were stained with lead hydroxide and sandwiched with carbon or formvar films before examination. General Abbreviations: BM *basement membrane;* EN *endothelium;* EP *epithelium;* L *capillary lumen.*

Figures 1 and 2

FIGURE 1

Wall of blood capillary in a skeletal muscle (rat). The lumen, which contains precipitated plasma proteins, is marked L *and the pericapillary space* PS. *The capillary wall consists of an inner or endothelial tunic (*EN*), a middle tunic or basement membrane (*BM*), and an adventitial tunic represented here by a few collagen fibrils seen in longitudinal (*cf_1*) or transverse (*cf*) section. Small vesicles in the cytoplasm of the endothelial cell appear aligned behind the luminal and tissue fronts of the cell. Some of them (*v_1*) are open to the cell surface and could be described as invaginations of the cell membrane; others (*v*) are closed and appear located deeper in the cytoplasm. In this case, the number of vesicles is greater on the tissue than on the luminal front of the endothelium.* × 73,000

FIGURE 2

General view of a transversely sectioned blood capillary in a skeletal muscle (rat). The lumen is marked L *and the pericapillary spaces* PS. *Parts of 3 endothelial cells* EN_1, EN, EN_3 *form the inner tunic at this level; their junctions appear at* j_1, j *and* j_3. *The nucleus of one of the endothelial cells can be seen at* n.

The middle tunic or basement membrane is marked BM. *It is relatively well outlined toward the endothelium from which it is separated by a shallow subendothelial space (*ss*) and frays into distinct fibrils (*tf*) towards the pericapillary spaces.*

*The adventitial tunic is represented by part of a pericyte (*P*) characteristically enwrapped between 2 leaflets of the basement membrane.* × 35,000



Figures 1 and 2 *(See legend on oppo ite page)*

tissue front of the cell (fig 3), and a few simple computations indicate that the vesicles represent a considerable amount of membranous material about 2 μ of membrane behind each μ of cell front and account for a sizable part of the total volume of the cell ~ 1/3

The spectrum of appearances encountered could be explained by assuming that the vesicles are formed by invaginations of the cell membrane that are pinched off and become closed elements in the cytoplasm carrying an imprisoned droplet of extracellular fluid A reverse process would evidently produce the same series of appearances but this time the sequence would start with a fluid filled vesicle in the cytoplasm which moves to the surface where its membrane coalesces with the cell membrane and where its content is discharged in the extracellular medium by the orifice created at the site of the coalescence If the 2 processes are combined the vesicles could transport fluid from one front of the cell to the other in small more or less equal portions or quanta The diameter of such a pocket is ~ 6?0 to 7?0 Å and its volume ~ 100 000 mμ^3 to ~ 180 000 mμ^3

This hypothesis has been tested experimentally but before presenting the appertaining results I should like to close the morphologic inquiry by reviewing information obtained on the cell junctions and on the basement membrane in this type of capillary At the level of the cell junctions (fig 4) there is a narrow intercellular space ~ 100 Å that separates 2 symmetric densifications of the apposed cell membranes The latter are sometimes backed by a condensation of the subjacent cytoplasmic matrix What seems to be important is the fact that the narrow intercellular gap is occupied by a material of moderate density frequently condensed into a denser intermediary layer or lamina In other words the narrow intercellular gap is not an unobstructed passage from the lumen to the pericapillary spaces The material in the gap does not represent however the cement substance postulated by light microscope studies That cement was supposedly characterized by its ability to reduce silver ions to metal and become impregnated by it In electron microscopy the silver deposits appear spread over a broad band centered on the junction but preferentially concentrated within the zones of densification of the adjacent cytoplasm [13] The presence or absence of pores in the intercellular spaces will be discussed later

The second layer the middle tunic of the capillary wall is represented by the basement membrane which appears as a continuous

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Figures 3 and 4

FIGURE 3

Gr i ing ection of a bloo l capillary of the myocardium (rat) shou ing the large number and irr gular distribution of vesicles (v_1 v) *on the tissue front of the endothelium The stoma' of some of these vesicles i e their opening to the cell surface shows clearly as a light circular area* (v)

The basement membrane appears as a densely matted felt of fine fibrils (f) *with sparse lightly thicker fibrils in its peripheral layers* (tf) *A few collagen fibrils of the adi ntiti are visil le at* cf *A ljacent muscle fibers are marked* mf × ??,000

FIGURE 4 a and b

Epithelial cell jun ti ns in blood capillaries of skeletal muscle (rat) The thi kening of tl e apposed cell membranes is vi ible in Fig 4b in between the short arrou The companion densification of the subjacent cytoplasm hows more clearly in Fig 4a at d *The intercellular space* (is) *is occupi d by a materi l of higher densit than that filling the peri apillary spaces*

Note in both ca es the oblique or si usoidal cour e of the junctions the pseudopodia (ps) *tl at flank tl em on the luminal front and tl e fact tl at the l a ement n mbrane pas es without interruption or infolding over the junct on (long arrows) The fibrill r texture of the basement membrane can be distinguishe l at* f *Collagen fibrils are marke l* cf × ?1,000



Figures 3 and 4 *(See legends on opposite page)*

coat of moderately dense material 200 to 500 Å in thickness. The limits of the coat are rather sharp toward the endothelium, from which it is separated by a narrow subendothelial space, and more poorly outlined toward the pericapillary spaces. In this direction the basement membrane comes in contact with various adventitial elements—collagen and elastic fibrils and cells of varied type: pericytes, macrophages, fibroblasts, and others.

In view of the tenuity and moderate density of this basement membrane one may wonder whether we are dealing here with an independent structural element of the wall or merely with a condensation of the ground substance of the connective tissue which could disperse easily once the endothelial substrate is removed. The answer is found in damaged specimens which show extensive retraction of the endothelium; although left behind, the basement membrane subsists as a distinct layer; apparently it is cohesive enough to resist the various mechanical and chemical insults involved in our preparation procedures.

In specimens fixed by osmium tetroxide the basement membrane appears as an amorphous layer of more or less homogenous density. In preparations stained by heavy metals, however, fine fibrillar elements of higher density can be demonstrated therein (fig. 5). In fact 2 types of fibrillar elements can be recognized: one finer and tightly meshed in the inner parts of the layer, another coarser, less abundant, and less intertwined in the outer parts of the structure. Grazing sections through the capillary wall are particularly favorable for demonstrating these fibrillar components, both of which appear to be distinct from mature collagen fibrils: they are thinner and do not show the characteristic periodic pattern of the latter. The coarser of the fibrils found in the basement membranes look morphologically similar to a special type of fibril usually encountered around elastic fibers. Needless to say, the chemical nature of all these fibrillar elements is unknown.

On the strength of this evidence we can conclude that the basement membrane is a felt of fine fibrils and that the meshes of the felt seem to be filled by another material, a matrix, which still appears amorphous at the present level of resolution.

So this is the type of construction encountered in most capillaries: between the blood plasma and the interstitial fluid, in interposed a succession of barriers consisting of a continuous endothelium, a continuous basement membrane, and an adventitia that, in this case, is discontinuous enough to be negligible as a true barrier.

Tracer Experiments on Blood Capillaries in Striated Muscles

What is the functional role of these layers in blood tissue exchanges? What structures participate and what mechanisms are involved in the transport of various substances across the wall?

Figures 5 and 6

Figure 5

Section through a bent blood capillary of the myocardium (rat). The lumen, cut in 2 places (L_1, L_2), is marked by colloidal gold particles. The endothelial cytoplasm contains small vesicles (v) and RNP particles (r). In the middle of the field a grazing cut through the basement membrane exhibits its poorly resolved, felt-like texture (f). × 82,000.

Figure 6

Blood capillary of the myocardium (rat) 10 minutes after an intravenous injection of colloidal gold (90 mg in 1.5 ml). The lumen (L) is marked by gold particles (g_1) which are also present in the endothelium—within small vesicles (g_2, g_3), in the basement membrane (g_4), and in the pericapillary spaces (g_5). Note the sharp decrease in particle concentration from the lumen to the endothelium, and the fact that within the latter the tracer is restricted to vesicles and does not have access to the cytoplasmic matrix. At the level of this section the endothelium is covered by the long process of a pericyte (P). 73,000.



Figures 5 and 6 *(See legends on opposite page)*

To find an answer to such questions we injected into the general circulation a tracer small enough to give meaningful information and dense enough to be seen individually in the electron microscope. Ferritin molecules ~ 100 Å in diameter[14] and micelles of colloidal gold ranging from 30 to 250 Å[1] proved to be useful for this type of work. At intervals ranging from 2 to 60 minutes after the injection of the tracer into the general circulation specimens for electron microscopy were collected from the heart or tongue to take advantage of the high concentration of capillary vessels in the muscle of these organs. Colloidal gold particles were found in large number in the lumen and in considerably smaller numbers in the endothelium, basement membrane and pericapillary spaces (fig. 6). In the endothelium they were as a rule restricted to the vesicles described in the vicinity of the cell membrane, the only other structure in which they were encountered being the so-called *multivesicular bodies*. Only occasionally were particles found in the cytoplasmic matrix (fig. 7). Since a relatively large number of micelles were detected in the pericapillary spaces it can be concluded that they had been ferried across the endothelium by vesicles. In these experiments only an occasional or no accumulation of particles was found against the basement membrane, apparently it allowed practically all micelles that crossed the endothelium to pass. After longer time intervals, one hour for instance, the situation was comparable except that most of the particles in the pericapillary spaces were found ingested by macrophages. It should be stressed that no tracer was found at any time in the intercellular spaces of the endothelium. The results obtained with ferritin molecules were less clear cut.[14] Frequently the tracer was found restricted to vesicles within the endothelium but sometimes ferritin molecules occurred freely dispersed in the cytoplasmic matrix with no indication of how they reached this location.

On the strength of the results obtained with colloidal gold it can be concluded that the *vesicles of the endothelium do function in transendothelial transport*. It is clear that they ferry the marker across the endothelium and it is highly probable that, together with

Figures 7 and 8

FIGURE 7

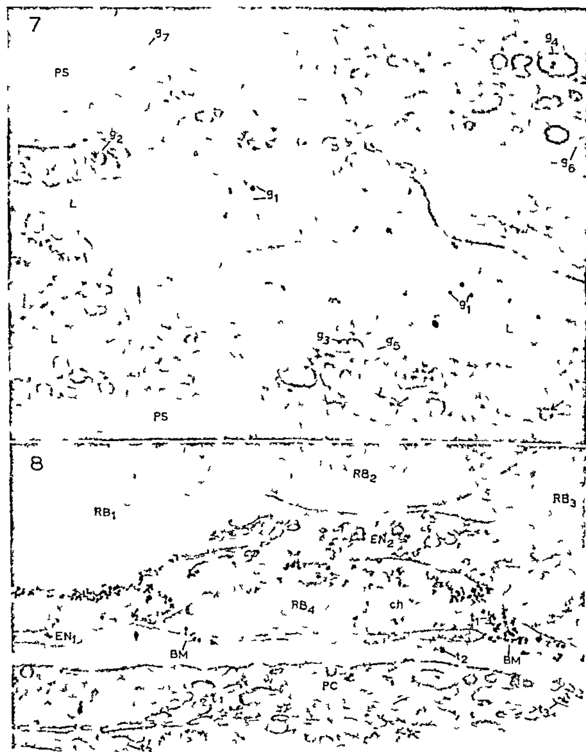
Blood capillary of the myocardium (rat) 10 minutes after an intravenous injection of colloidal gold (90 mg in 1.5 ml.). The irregular lumen is marked by circulating particles (g_1). *The endothelium contains a number of similar particles enclosed singly* (g_2, g_3) *or severally* (g_4) *into vesicles. One tracer particle* (g_5) *appears against a slightly dense patch which could correspond to the top of a sectioned vesicle; another particle* (g_6) *is definitely free in the cytoplasmic matrix. A few tracer particles* (g_7) *have reached the pericapillary spaces.*

Note that many other vesicles (arrows) contain small particles that seem to be attached to the inner surface of their membrane. Their identification as gold colloidal micelles is uncertain because their density is low and their form irregular. × 77,000

FIGURE 8

Small blood vessel (venule) in the cremaster of a rat 2.5 minutes after a local subcutaneous injection of histamine. The tracer, colloidal mercuric sulfide, was previously injected in the general circulation.

The lumen is occupied by 3 erythrocytes (RB_{1-3}). *The endothelium* (EN_1, EN_2) *is discontinuous through the gap marked by arrows; tracer particles* (t_1), *chylomicrons* (ch) *and a red blood cell* (RB_4) *have penetrated and dissected the wall up to the basement membrane* (BM) *which in this case appears particularly thin and poorly outlined. Note that most of the particles are retained by the basement membrane and that the intramural deposit is highly concentrated presumably as a result of water and solute escape through the basement membrane. Note also that of the few particles which have reached the pericapillary spaces* (t_2), *some have already been incorporated* (t_3) *by a phagocytic element* (PC). × 16,000



Figures 7 and 8 *(See legends on opposite page)*

the marker they transport water and solutes since there is room enough for a few hundred thousand molecules of corresponding size in addition to the tracer particle in each vesicle of 650 Å diameter If this modality of transport seems to be well established by our experiments it should be pointed out that its relative importance in the overall exchanges between blood and tissue fluids remains unknown transport by vesicles may account for all or only for part of these exchanges and only future quantitative work will tell us whether we are dealing here with the main mechanism of transport or with an accessory one of limited importance

In any case it should be realized that transport across the endothelium represents only one step in the entire operation There is a second barrier to be crossed the basement membrane and if in the experiments so far reported it did not markedly affect the passage of the tracer this does not mean that it will behave in the same way under different conditions or in respect to other tracers In fact there is good ground to assume that the basement membrane should play an important role in such exchanges the principal reasons being the following it is difficult to ascribe specificity to a transport in quanta and moreover there are types of capillaries in which the endothelium is discontinuous or fenestrated and in which the blood plasma gains direct access to the basement membrane which appears to be the only continuous barrier in the wall of the vessels

Tracer Experiments on Renal Glomerular Capillaries

Capillaries with a fenestrated endothelium are encountered in many viscera[8, 16] and the fenestration of their endothelium becomes particularly extensive in the glomerular capillaries of the kidney In addition to an extensively fenestrated endothelium these capillaries are characterized by the existence of a third cellular layer—made up of the pseudopodia of the visceral epithelium Their basement membrane is also thicker and apparently more substantial than in other capillaries visceral or somatic Glomerular capillaries represent a favorable object of study because in their case we are dealing with one way transport only—i e from the lumina to the capsular space—and because the nature of the capsular fluid is relatively well known It has been collected by direct micropuncture in a number of species and its analysis has shown that it is a protein free or almost free filtrate of the plasma[cf 1]

In the experiments on glomerular capillaries carried out in collaboration with Drs M Farquhar and S Wissig the tracers used were again ferritin and colloidal gold[18-20] After short time intervals (3 to 15 minutes) the ferritin was found in high concentrations in the lumen From the lumen it appeared to gain free access, through the fenestrae of the endothelium to the basement membrane Within the latter the tracer was found in noticeably lower concentrations and more or less evenly distributed in surface and in

Figure 9

Renal glomerular capillary of a nephrotic rat one hour after the intravenous injection of [illegible]0 mg ferritin

The basement membrane (BM) *crosses obliquely the field separating the epithelium* (EP) *at left from the endothelium* (EN) *at right The lumen is hardly visible at the extreme right Large deposits of ferritin infiltrate the spongy areas* (sa) *and the luminal layers of the basement membrane Such deposits identify the basement membrane as the main filtration barrier Fewer particles penetrate the peripheral layers of the filter and reach the epithelium where they can be seen in invaginations of the cell membrane* (t_1) *in closed vesicles and small vacuoles within the cytoplasm* (t_2) *and in dense bodies or absorption droplets* (t_3)

At this relatively late time point the endothelium contains membrane bound vacuoles (pb) *filled with packed ferritin—an indication of the phagocytic activity by which the filtration deposits are removed* × [illegible]3 000



Figure 9 *(See legend on opposite page)*

depth Few ferritin molecules reached the foot processes of the epithelial layer, and those that did appeared to be caught either in small invaginations of the cell membrane or in small vesicles within the cytoplasm After longer time intervals (30 to 60 minutes), 2 new noteworthy features emerged first there was a gradual increase in the concentration of the marker in the luminal strata of the basement membrane and finally extensive piling up of ferritin molecules against its luminal surface second the number of ferritin molecules captured by the epithelium increased and in addition to those located in membrane invaginations and small vesicles in the foot processes the tracer appeared in the cell body proper in large vacuoles and in dense bodies

To confirm these findings, we also administered the tracer to rats rendered nephrotic by treatment with the aminonucleoside of puromycin [1] In such cases the permeability of the glomerular capillaries is increased and substantial amounts of blood proteins are lost during glomerular filtration In nephrotic animals the piling of ferritin against the basement membrane was about as striking as in normals after long time intervals (fig 9) but the amount of ferritin in the basement membrane and in the epithelium was considerably greater (fig 10*) The epithelium showed its typical response to the nephrotic condition the extensive disappearance of the foot processes In these altered epithelial cells as in normal ones the marker was restricted to membrane limited spaces i e vesicles vacuoles and dense bodies In nephrotic animals there was a definite reduction in the extent of the fenestration of the endothelium

From these experiments it can be safely concluded that in this type of capillary the main filtration barrier is the basement membrane The tracer is retained by it as by a filter It is clear however that the filter is imperfect Even under normal conditions it leaks a detectable amount of the tracer which is recovered at least in part from the filtrate by the epithelium that seems to function as a monitor of the filter proper As expected, operations connected with this recovery are considerably enhanced when the filter becomes more leaky in nephrotic animals Turning now to the filter proper it should be pointed out that the ferritin molecules that escape through it were found distributed at random within the membrane There was no preferred relationship to the slits of the epithelium for instance Moreover and probably more important no pores were seen in the basement membrane The tracer molecules were found embedded in its substance without channels ahead or trails behind Pores of simple geometry allowing the passage of a ferritin molecule would have a diameter in excess of 100 A and should be visible Since they are not we are led to conclude that they are either extremely tortuous and consequently difficult to see in sections of the thickness used (~ 500

*Figure 10 reproduced from Farquhar M C and Palade G F Segregation of ferritin in glomerular absorption droplets J Biophys & Biochem Cytol 7 9 1960 By permission of the Journal of Biophysical and Biochemical Cytology

Figure 10

Part of a renal glomerulus in a nephrotic rat 2 hours after the intravenous injection of 50 mg ferritin

Most of the field is occupied by part of an epithelial cell (FP) which contains the marker (ferritin molecules) in small vesicles (t1) small vacuoles (t2) large vacuoles with a light content (t3) structures of intermediary appearance (t4) and—finally—typical dense bodies (t t6 t7) It is assumed that all these forms represent progressive stages in the segregation and concentration of the marker and other materials incorporated by the cell from the glomerular filtrate

The basement membrane (BM) of the capillary covered by this epithelial cell appears in the upper left corner infiltrated by numerous ferritin molecules

The lumen is not visible in this field but some urinary spaces can be seen (US) × 73,000 (From Farquhar and Palade J Biophys & Biochem Cytol 7 1960)

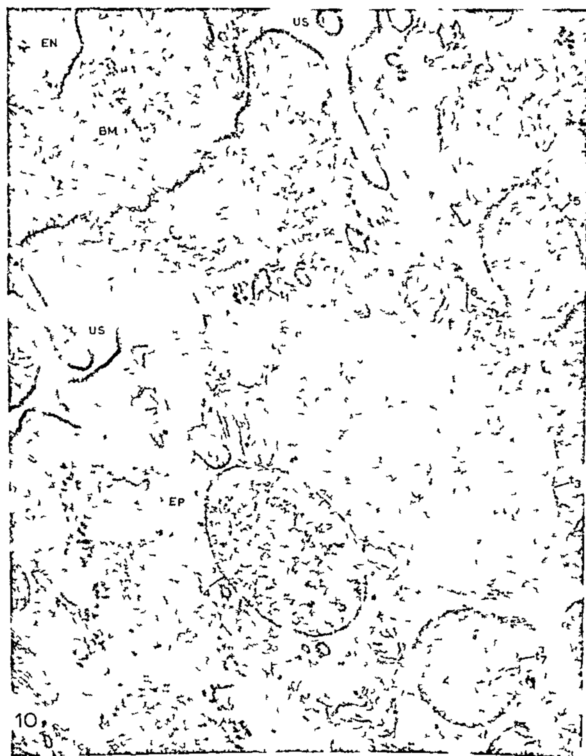


Figure 10 *(See legend on opposite page)*

to 800 Å) or that permanent pores do not exist One has the impression that the marker moves through a yielding gel creating a channel as it moves As far as the activities of the other layers are concerned they appear to be ancillary if we restrict our interest to the filtration process proper and disregard morphogenetic processes for the moment The endothelium seems to function as a valve that regulates the amount of plasma which gains direct access to the filter whereas the epithelium behaves like a monitor which partly compensates for the imperfections of the filter Analogies with the endothelium and the adventitial cells (primarily macrophages) of muscle capillaries are quite evident

Effects of Histamine and Serotonin upon the Structure of Small Blood Vessels

In the light of these experiments on glomerular capillaries should we conclude that the basement membrane is also the main filter in muscle capillaries i e in capillaries with a continuous endothelium and that in the latter the vesicles represent only a more refined valve than the fenestrae of the glomerular capillaries?

The last experiments to be reported suggest that such a conclusion is justified at least in part In collaboration with Dr Guido Majno and Miss Gutta Schoefl [3] from the Department of Pathology at Harvard we tried to find out what changes are introduced in the structure of blood capillaries by local histamine or serotonin treatment which is known to increase the permeability of small vessels markedly [4] The experimental device was simple and relied primarily on the discovery of a favorable specimen the cremaster of the rat a thin layer of striated muscle—2 muscle fibers thick—located under the skin of the scrotum The marker this time a coarser colloidal particle of mercuric sulfide (200 to 500 Å in diameter) was introduced into the general circulation and histamine or serotonin in doses of 50 μg and 5 μg respectively was injected locally between the skin and the cremaster The latter was excised and fixed at various time points thereafter either in toto for light microscopy or in small blocks for electron microscopy The effect of the 2 amines becomes visible under the dissecting microscope in approximately 3 minutes and spectacular in 10 to 15 minutes It consists in a spotty blackening of the vessels that otherwise retain their usual appearance at low magnifications The electron microscope provides a satisfactory explanation for the blackening The "lesion" produced by the amines is a local discontinuity in the endothelium apparently caused by the pulling apart of 2 endothelial cells over longer or shorter distances A gap is thus produced through which the plasma loaded with the marker gains direct access to the basement membrane The latter apparently lets the water and many of the solutes pass but as a filter retains the marker In time relatively large deposits of HgS particles accumulate within the wall of the vessel and start to dissect its layers I should point out that the amines affect preferentially the small venules as clearly indicated by the examination under the light microscope of cremasters mounted in toto [3] but the lesions extend on the vascular tree toward the capillaries at least toward their venous ends In view of the preferential localization of the lesions in itself a very intriguing finding it is *not surprising that the morphology* of the affected vessels is sometimes extremely complex Successive and unequal deposits are found in the thickness of the wall cleaving its various layers which in the case of a venule can be more numerous than in the case of a capillary In addition to the marker many other circulating particles are retained by the filter Frequently chylomicrons accumulate within the wall together with deposits of proteins some of which polymerize into tactoids with the periodicity of fibrin Finally cellular elements i e platelets erythrocytes and leukocytes find their way into these dissecting aneurysms of the wall of the vessels What is remarkable is the fact that with all its tenuity and poor definition the basement membrane of these vessels is capable of retaining the large deposits formed by the residues of filtration Relatively few particles reach the pericapillary space and those that

do are rapidly picked up by phagocytic elements located along the vessels (fig 8) The effect of histamine on the structure of blood capillaries has been surveyed by Alksne [6] who arrived at the conclusion that the amine increased the pinocytic activity of the endothelium in addition to causing the formation of channels across the endothelial cells The differences in results and interpretation between his and our experiments are due to differences in specimens and time points examined In his case the specimen (skin) was less favorable and the timing inadequate The results of the histamine and serotonin experiments indicate clearly that the basement membrane of the venules and capillaries of the cremaster behaves when denuded like that of glomerular capillaries it proves its ability to function as a filter by accumulating a conspicuous filtration residue Yet it is too early to conclude that the endothelium does not screen what it transports at all for so far only a very small number of various kinds of particles have been tested

General Comments

Admittedly much remains to be done before the relation between the structure and the function of various capillary vessels is clearly understood but the findings so far recorded already suggest distinct roles for the successive layers of the capillary wall and point to the basement membrane as a functionally important component This layer appears to be the best candidate for the role of selective filter It remains to be seen however whether its selectivity can be explained by simple devices such as pores of fixed geometry or by more complex properties In this respect the higher permeability of all capillaries to lipid soluble substances (*when molecules of similar diameter are considered*) should not be forgotten With this in mind it is to be regretted that we know so little about the chemistry of the filter Whatever we know is derived from histochemical tests which indicate that the basement membrane consists of a mucopolysaccharide [7] probably conjugated or associated with proteins More knowledge will undoubtedly be helpful I hope that the work presented may act as a stimulus in this direction

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can incur some debt Perhaps, the extraordinary number of mitochrondria in cardiac muscle may improve its efficiency in meeting its energy requirements which are continuous

Dr Fishman I should like to direct a question to Dr Palade Although your presentation this morning indicated that the capillary endothelium may be continuous or discontinuous it made no reference to pores in the basement membrane these pores are generally held to be involved in the transport of fluids across the capillary walls Quanta seemed to replace pores for this movement of fluids But by what mechanism do gases move across myocardial capillaries

Dr Palade There would be no problem there because I suppose gases move very easily across the cell membrane and probably can move easily across the basement membrane

Dr Fishman You believe then that water crosses the capillary wall by a different route from that traversed by gases?

Dr Palade What we are proposing is simply a discontinuous channel for a continuous channel We are also pointing out that in addition to discontinuous or continuous channels in the endothelium diffusion or exchange across the basement membrane must still be taken into account

It is easier to account for the movement of larger molecules by this mechanism than of water As Dr Fishman has intimated this is a mechanism geared particularly to exchanges in which large particles or molecules are involved other mechanisms involving pores have to be postulated for the mechanism of water In any case if the pores do exist they are below the practical limits of resolution of the microscope i e below 20 or 15 Å The dimensions postulated have not been seen and again they should have been seen

Dr Fishman May I close this by asking do you visualize any rule for pinocytosis in this transport mechanism?

Dr Palade What I have described is somewhat comparable to pinocytosis The only difference is that instead of getting large and more or less unequal particles picked up by the cell—i e droplets of fluid picked up by the cell—the vesicles are submicroscopic

Dr Milton Landowne (Baltimore, Maryland) Would you comment on the possibility that the sections are actually those of highly tortuous channels rather than vesicles?

Dr Palade This can be answered by studying serial sections, and serial sections show that the large majority of these vesicles are independent structures—that they are not part of a continuous system of tortuous channels

Dr Rhodin In this connection, I should like to ask Dr Palade if he thinks that the entire fluid transport occurs via these vesicles? Studies of the kidney have led us to believe that the intercellular space is utilized for the transport of fluid Would this also apply to these capillaries?

Dr Palade I can only restate what I have already said The markers that we used are not found at the level of the intercellular spaces Some of these markers i e the colloidal gold particles are very small—of the order of 30 or 40 Å Therefore they would have access to pores if pores of the order of 60 Å do exist at the level of the cell junctions or at the level of the so called cement substance

Dr Rhodin Dr Huxley I should like to ask if you have been able to see the fine cross bridges between the myofilaments which you demonstrated so beautifully here in preparations other than the glycerinated muscle? I am not questioning your results but it would be pleasant to find them in ordinary fixed muscle

The reason for bringing up this question is the knowledge of the results obtained by Sjostrand and Andersson Cedergren (*J Ultrastructure Res* 1 74 1957) who suggested that each filament is composed in turn of subfilaments—unit filaments—arranged in a helical or perhaps in a coiled fashion

Dr Huxley We can see the cross bridges in preparations of frog sartorius muscle for instance or of one of the toe muscles They do not show up so clearly as they do in the

glycerinated muscle, but they can be seen quite distinctly As a matter of fact I think they are visible in a picture of live rabbit sartorius muscle in a paper which I published in the *Journal of Biophysical and Biochemical Cytology* You can see them quite clearly in insect flight muscle that has not been glycerinated

Dr Rhodin Would you like to comment on the views of Sjostrand? Do you think a coiled structure does not exist in these filaments?

Dr Huxley Well I think that the cross bridges we have seen seem to project out from the myosin filaments at regular intervals along the length of the filaments and point toward each of the 6 surrounding actin filaments in turn as though they were located on a helical course This pattern as well as the tapered appearance of the filaments at their ends would fit in very nicely with the idea that the bridges were made up of smaller subunits myosin molecules with from 10 to 20 myosin molecules lying side by side within the diameter of each filament and many in series along its length

Dr Weidmann Dr Rhodin did you really expect conduction velocity to increase with an increasing number of intercalated discs per unit length other things being equal?

Dr Rhodin Yes

Dr Weidmann I cannot quite follow the argument If you increase the number of discs per unit length the electric resistance inside the fibers can only increase And according to cable theory the propagation velocity then drops Even if you assume something like a functional transmission from 1 cell to the next I have difficulty in seeing how a higher number of such sites should speed up conduction Propagation across more synapses can only be associated with more delay

Chairman Kossmann Dr Rhodin do you wish to comment on this?

Dr Rhodin Not at the moment

Dr Hoffman I have 2 questions In the capillary the basement membrane seems to be a diffusion barrier I wonder if the outer layer of the sarcolemma which is not continuous with the sarcoplasmic reticulum as far as I know might be analogous to the basement membrane It could thus be our desired diffusion barrier whereas the inert layer of the sarcolemma which is continuous with the intracellular structures might perhaps be a structure of very high permeability? Is there any reason to consider the muscle membrane this way?

Dr Fawcett I will simply say that the muscle fibers in cardiac muscle do have an extraneous coating that in all respects resembles the basement membrane of the capillaries

Dr Palade This outer layer is similar to the structure of the basement membrane and to the periphery of every muscle fiber in the skeletal musculature as well as in the myocardium It is different from the plasma membrane It can be removed from the plasma membrane It may definitely act as a tissue barrier for large particles as in the case of the blood capillary

Dr Podolsky I should like to ask Dr Huxley how he managed to get the 2 sets of thin filaments to slide past each other Couldn t they have coiled first and then slipped past after the contraction was over?

Dr Huxley That preparation was glycerinated muscle that was shortening in ATP under load After it had shortened down the required amount the ATP was washed out of it so that instead of going into the relaxed state after the contraction was over it went into a state of rigor

Now on any of the models even ones depending on a coiling of the actin filaments where you envisage an interaction of the cross bridges with the actin filaments you would think that when a glycerinated muscle came to the end of its shortening and the ATP was washed out the bridges would become attached permanently to the actin filaments instead of repetitively this attachment would hold the actin filaments in the position they occupied while the shortening was going on Then there would be no opportunity for them to uncoil again—to straighten out—in the center of the sarcomere as Dr Podolsky suggests

I agree that this type of observation on a

living muscle during contraction would be more difficult to interpret But in this case the effect you see may be significant

Dr DeHaan Dr Rhodin it has been pointed out that one of the differences between myocardium and the specialized system that Dr Rhodin has just presented is the lack of a basement membrane in the conduction system Have you seen this difference?

Dr Rhodin There is a difference In the myocardium as well as in the S A and A V nodes each cell is surrounded by a basement membrane This membrane is continuous at the site where the cells adjoin that is at the intercalated disc However in the common bundle and its branches the entire set of cells in each tiny bundle—3 to 4 cells in width—usually is surrounded by the basement membrane so the functional units in the fiber are all surrounded by basement membranes

Dr Hoffman Dr Rhodin, where were your sections of the A V node from?

Dr Rhodin The ones that I described as being A V nodal fibers were taken from the approximate middle of the node Of course as you approach the common bundle there is a direct continuity between the nodal fibers and the common bundle fibers

Myocardial Metabolism—1924

When a muscle contracts tension is developed and external work is done if the tension is made use of to raise a weight or perform other functions requiring expenditure of energy It is obvious therefore that there must be something in resting muscle which possesses potential energy of some kind and that on excitation some change takes place in this system resulting in loss of potential energy We know that lactic acid is formed and that the actual contractile process is not associated with the giving off of carbon dioxide nor with the consumption of oxygen It is not in fact an oxidation so that the inogen conception fails here Although there must be some large molecules or aggregate containing the lactic acid group these cannot be of a protein nature with intramolecular oxygen as one side chain and an oxidisable group at another place At the end of the contraction the cell machinery possesses less potential energy and the systems actually participating in the change inogens if we use Hermann's name though not exactly in his sense have let loose lactic acid

Now to restore the system to its original state with increase of energy content a further exothermic reaction is necessary In his process the system is restored to its original state of high potential energy so that the reaction by which it is effected must be one in which a considerable amount of energy is set free This is shown by the large consumption of oxygen and liberation of carbon dioxide indicating oxidation of some combustible substance We have seen already that no nitrogen metabolism is associated with muscular work as such the oxidised substance must therefore be carbohydrate or fat It appears that carbohydrate is normally used in the muscle itself but fat appears also to be capable of serving the purpose perhaps indirectly in the intact animal—W M Bayliss *Principles of General Physiology* Ed 4 London Longmans Green & Co 1924

II Biochemistry

Chairman: John V. Taggart, M.D.

Introduction

By John V. Taggart, M.D.

IN THIS morning's session, a great deal was said about ultrastructure in the myocardium, notably concerning the myofilaments, the endoplasmic reticulum and the mitochondria, as well as the capillaries. We now know that many of these structures possess specific biochemical and enzymatic properties.

This afternoon's program will deal with selected metabolic features of the myocardium. In an effort to gain greater insight into the relationship between structure and function, we shall be concerned mainly with the so-called mechanochemical transformation that underlies muscle contraction, that is, the conversion of chemical energy into mechanical work. As has been pointed out by others, the study of muscle contraction follows many paths and involves a variety of experimental technics. One of these is the analysis of structure, about which we have already heard. Another is the characterization of the component proteins of these structures. The third deals with the enzymatic activities of the proteins and the fourth with a consideration of theoretical model systems. Finally, we shall return to the physiology and metabolic regulation of the intact tissue.

Because one of the reactions most frequently implicated in the process of muscular contraction is the hydrolysis of ATP, it is appropriate that we begin on this note.

From the Department of Medicine, College of Physicians and Surgeons, Columbia University, New York, New York.

Biological Thinking and Investigation

Yet I feel [illegible] of two broad principles which should govern all biological thinking [illegible] investigation that I should be lacking in courage and honesty were I to neglect to bring them before you. The first is that there is no limit to the degree to which the mechanisms of life can be explored and elucidated by what we commonly call physical [illegible] chemical means: nature has erected no barrier beyond which trespassers will be prosecuted, and arranged no [illegible] into which it is not profitable to go. The second is that however far we get we shall still find function, adaptation, organisation, purpose in the processes we explore: the purpose may seem queer sometimes, the function may be unexpected, the adaptations may prove imperfect, but viewed as a whole they will be found to represent the best compromise available. —A. V. Hill: *Living Machinery*. New York, Harcourt Brace & Co., 19[illegible], p. [illegible]8.

The ATPases of Muscle Proteins

By Manuel F Morales, Ph D and Shizuo Watanabe, Sc D

The ideas and results of Blum Oosawa Strohman and many others are reviewed and when consolidated with the authors work lead to the following picture of muscle protein adenosine triphosphatases (ATPases) Two major features of myosin ATPase seem to be a catalytic interaction between enzyme a metal cation and the terminal pyrophosphate moiety of adenosine triphosphate (ATP) and a rate retarding interaction between enzyme Mg and the purine ring of ATP Sulfhydryl groups of the enzyme participate at both loci In the catalytic interaction an ionizable group (pK ca 6 8) may participate G actin molecules binding ATP (probably by the purine ring) and relieved of their mutual repulsion cooperate in catalyzing dephosphorylation thereby giving rise to the somewhat loose possibly helical structure of F actin

IN CONTRIBUTING to this symposium we are tacitly assuming that as regards our subject there is no substantial difference between heart and skeletal muscle tissue It has also to be said that although ATPases are emphasized in the title and in the paper itself it is very possible in view of work such as Strohman s[1] that in situ one or more of these enzymes function in phosphate transfer rather than in ATP hydrolysis Such a possibility would not vitiate the merit of ATPase studies since the objectives in most of these studies are clues about enzyme surfaces and enzyme substrate interactions For these purposes it is quite legitimate to consider various acceptors among them water At the same time we must acknowledge that even if these studies were totally successful and we understood precisely the mechanisms of the enzymes we would still have to integrate our information with that of other speakers in order to understand how muscle 'works "

In this paper we shall consider primarily the ATPase activities of myosin and actin but first it is convenient to touch upon some properties of ATP and its relatives

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Substrates

For the enzymes to be considered the natural substrate appears to be adenosine triphosphate (ATP) A long known property of ATP is that its free energy of hydrolysis is substantial A possible consequence of this fact for interactions of ATP with its enzymes is the plausibility of formation of energetically costly enzyme intermediates Other conceivable properties of ATP as a substrate such as charge and binding affinities of its various moieties have generally received scant attention In recent years however interest in ATP metal cation complexes has been kindled by mounting evidence that metal cations play a critical role in ATPase and contractile properties

It is safe to assume that metal cations are chelated by the polyphosphate end of ATP and may form a sort of electrostatic ' cement linking substrate to enzyme and facilitating distal hydrolytic attack by water molecules However enzymologic investigations have prompted speculation that metal cations also interact with the ring end of ATP, either to anchor it to enzyme or to its own polyphosphate end The existence of these interactions can be and has been investigated in the absence of enzyme To establish further that these interactions are part of the "normal' enzymatic process—for example to show that the true substrate is a metal complex—is much harder it has not been done decisively

for either myosin or actin although most recently it has been argued for the related enzyme creatine kinase[2]

The thermodynamic affinity of metal cations for ATP (without regard for the structure of the complex) has been studied by several competent investigators and methods and it is therefore disturbing that the results have varied widely The hypothesis that the adenine ring participates in metal binding was first put forth by Blum[3] who sought to account for a ring enzyme anchor point and shortly thereafter by Levedahl[4] Szent Gyorgyi[5] and Eichhorn[6] all of whom noted that simultaneous chelation of metal ions by ring *and* polyphosphate moieties would stabilize ATP in a curled configuration of possible functional significance Although Levedahl s measurements[4, 7] can blend with such a hypothesis direct evidence for ring metal cation interactions has been wanting Recent preliminary observations[8] by K Hotta and J Brahms in our laboratory may help resolve the difficulties clouding this problem Hotta and Brahms found that if the affinity (formulated for a 1 nucleotide 1 metal cation complex) is measured at various concentrations of metal cation there is an inverse correlation between affinity and concentration This trend is also noticeable in the calculations of other authors who employed methods of varying sensitivity and therefore worked at different concentrations The implication is that not only a 1 1 complex but also a 1 2 complex exists Hotta and Brahms have not yet completed their study but for purposes of orientation we shall mention that at an ionic strength $[(CH_3)_4 NCl]$ of 0 05 M and total nucleotide and MgCl concentrations of 10^{-3} M and 10 M respectively K_{98} (ATP) = 8×10^3 and for inosine triphosphate (ITP) K_{98} (ITP) = 12×10^3 where K is the apparent 1 1 affinity in li/mole These values are from pH meter measurements in the neighborhood of pH 6 8 i e they reflect displacement by cations of protons attached to the polyphosphate structure Protons are also displaceable from the rings of ATP ITP and cytidine triphosphate (CTP) at characteristic pH s In these cases proton detachment (whether by reaction with OH^- or by cation displacement) is accompanied by measurable shifts in the ultraviolet spectra of the nucleotides and from these shifts one may infer the affinities of the cations for the *ring* moieties of the various nucleotides * Taking advantage of this effect Hotta and Brahms have found that for the rings K_{98} (ATP) = 1 6 × 10 li/mole and K_{98} (ITP) = 1 5 × 10- li/mole † They have also found appreciable ring binding with the diphosphates ADP and IDP but not with monophosphates ribosides or free bases Taken together these various observations are strong support for the general assumption that metal cations can interact with the ring atoms of nucleotides they also indicate that a (small) fraction of the nucleotide metal cation complexes are in the curled configuration However it remains for other experiments to decide whether these configurations have a functional significance (for instance for fitting into enzyme crevices)

In principle both gross and subtle properties of different portions of the ATP structure can be studied by observing the behavior of analogs (fig 1) Thus it has been established in a gross way that orthophosphate is hydrolyzed only from a triphosphate structure (e g ATP or tripolyphosphate PPP not ADP or pyrophosphate PP) In view of PPP the attachment of a purine or a pyrimidine ring and ribose to the triphosphate structure is inessential for myosin triphosphatase activity but it modifies such activity Since ribose triphosphate is not yet available one cannot decide whether the enhancement stems

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* It is logically possible though much less likely that the metal cation bound to the polyphosphate structure is not simultaneously touching the ring but only attenuating the electrostatic field that othe ise perturbs the ring protons

†Although the r ng affinites for ATP and ITP are about the same it should be recalled that around neutral pH (e g 8) the proton competition for the ATP ring site w ll be nil whereas the proton compet tion for the ITP r ng site will still be formidable accord ngly the fraction of r ng sites holding cation will be much greate in ATP than in ITP

Ring variations

Sugar variations

(Hasselbach) (Ikehara) (Ikehara) (Ikehara) (Ikehara) (Ikehara)

Polyphosphate variations

(Moos)

Figure 1

Some substrates for ATPase

from the ring from the ribose moiety or simply from the elimination of an extra nega tive charge in PPP From the analogs of Hasselbach[9] and Ikehara et al [10] it appears that considerable liberties can be taken with the ribose region without suppressing myosin activity in fact, it may be that reducing the enzyme ribose interaction enhances activity On the other hand the fact that *either* a purine or a pyrimidine moiety permits active catalysis suggests that specific and simultane ous interaction between several ring atoms and enzyme is not obligatory or that there is flexibility in the enzyme structure Another type of interesting analog adenyl methylene diphosphonate (adenyl PCP), was recently introduced by Moos et al [11] Even though iden tical with ATP in every steric respect adenyl PCP is peculiarly inert toward the muscle proteins In this molecule however oppos ing resonance is suppressed and probably causes the pK for the final phosphate ioniza tion to shift from 6 5 to 8 2 and reduces Mg^{++} chelation at neutral pH We are thus again reminded of the importance of the nucleotide charge for substrate capability

With the advent of diverse analogs such as those introduced by Ikehara et al and Moos et al a number of other deductions will un doubtedly be forthcoming An increasingly definite deduction from analogs already avail able will be discussed in the next section

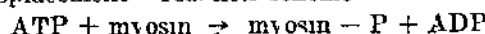
Myosin

Elsewhere in this symposium Olson and Bing and Kato discuss the physical proper ties of myosin but it is cogent for us to know now that to date the smallest kinetic unit re ported for solutions of purified *skeletal* myosin

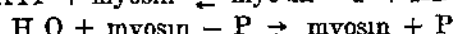
has a mass of not less than 4 × 10^{5} Gm Various attempts—the latest being that of Nanninga and Mommaerts[1] —to measure the myosin weight that combines with 1 mole of ATP or of PP have led to very nearly this same value Therefore although we are unconvinced* we have to agree that on present evidence the ATPase site of myosin seems to occupy but a tiny portion of the molecule Speculation about the function of this tiny site has been dominated recently by two ideas that we shall now consider

The Double Displacement Scheme

By analogy with certain other hydrolases there has been proposed (see for instance Weber[13]) for myosin a so called double displacement reaction scheme



$$ATP + myosin \rightleftarrows myosin - P + ADP$$



$$H_2O + myosin - P \rightleftarrows myosin + P$$

Justification of such a scheme has been attempted along 3 lines (1) According to the scheme a P^{32} label introduced in the form of ADP should back incorporate into ATP Although early European reports seemed to bear out this expectation it now seems very definite that the back incorporation is not observed with pure muscle proteins (2) Under appropriate conditions of isolation it ought to be possible to interrupt the reaction and to demonstrate enzyme with covalently attached phosphorus This has also been reported but such experiments are equivocal owing to possible sorption of inorganic phosphorus by the active enzyme Maruyama and Gergely[14] for instance have recently concluded that this sorption rather than any true phosphorylated intermediate explains this sort of experiment (3) It is possible that the myosin catalyzed excess incorporation of O^{18} from H_2O^{18} into inorganic phosphorus (produced from ATP) indicates a phosphorylated intermediate since it does not occur in either ATP or inorganic phosphorus However Koshland and his associates[15] who discovered the O^{18} exchange have shown since that its rate is sensitive to purine ring substituents so that for all practical purposes the exchange occurs while the ATP (or other nucleotide) is still intact and thus its existence is not evidence for a phosphorylated enzyme In summary experiments that with other enzymes have eventually demonstrated an intermediate have in the case of myosin been quite inconclusive thus far

Before attempting to discuss a second major hypothesis about the myosin ATPase mechanism it is convenient to consider the general classes of substances that modify this ATPase

Substances That Modify Myosin ATPase

Salts in General

In his pioneering work Szent Gyorgyi[16] showed that the chlorides of various metals had a diphasic effect on both the solubility and the ATPase activity of myosin At neutral pH low concentrations of salts precipitated the enzyme and activated ATPase while greater concentrations solubilized the enzyme and inhibited the ATPase Szent Gyorgyi felt that the solubility phenomena resulted from cation binding (which rendered the protein isoelectric) followed by anion binding (which charged the protein negatively) and he considered that although diverse cations catalyzed ATPase the precise effect of a particular concentration depended not only on the specific cation but also on the ionic strength Unfortunately these early discoveries and ideas have not been widely examined or pursued As regards ATPase there has arisen a complicated literature in which investigators report the pH the cation used and the ionic strength KCl has been regarded as an inert electrolyte that provides only ionic strength and the possibility that anions might have

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*Reasons for doubt are both subjective and objective Other enzymes once really purified have invariably proved to be of a smaller order of magnitude so it is puzzling to be faced with such an exception A more scientific doubt arises from the recent recognition that the binding of ATP to enzyme can be strongly inhibited by polyanions Investigators of site weight have borne in mind the possibility that the true substrate is an Mg ATP or Mg PP complex[12] so to reduce complications the system has generally been saturated with Mg salts sometimes with $MgSO_4$ Not only may the increased ionic strength have discouraged binding to enzyme but also SO_4 may have competed successfully for erstwhile ATP sites

specific effects has been largely disregarded thus a report may read "Mg^{++} (actually $MgCl_2$ or perhaps $MgSO_4$) is an ATPase inhibitor at ionic strength of 0 60 M (actually in 0 60 M KCl)" As a result of preliminary work with solvents more nearly "inert" than KCl we now know that such statements are treacherous since superimposed on the truly ionic strength effects of these electrolytes is a competition between K^+ and Mg^{++} (and no doubt H^+) for specific binding sites and also specific Cl^- and $SO_4^=$ binding which may be competitive with ATP binding[2] Nevertheless the statements that follow are tentative because our work has not been extended to a sufficient number of ions As regards solubility it appears[17] that the precipitation when electrolyte is first added is predominantly a nonspecific ionic strength—presumably screening of solubilizing repulsion—effect The subsequent solubilization however is as Szent Gyorgyi surmised the result of anion binding and the higher the anionic valence the greater the solubilization per mole of anion As already anticipated by Mihalyi[18] Cl^- is certainly bound such anions as sulfate ferricyanide and pyrophosphate are even more strongly bound As in the creatine kinase system acetate seems fairly "inert" Among the cations we have reason to think that tetramethylammonium is also "inert" (see below) Our best current approximation to an inert electrolyte is therefore tetramethylammonium acetate however because of its ready availability we have also used tetramethylammonium chloride (TMAC) especially for low ionic strengths At an ionic strength of a few tenths TMAC myosin ATPase is soluble but essentially inactive If now a metal chloride is added at increasing concentration (but still low enough so as not to change the ionic strength markedly) the ATPase activity rises to a maximum level and stays there As would be expected from all past work $CaCl_2$ gives the highest asymptotic activity of the simple salts thus far tested the activity provided by KCl however is appreciable Used in this way $MgCl_2$ is definitely an activator although much weaker than $CaCl_2$ The particular activity attained with a salt is undoubtedly the resultant of co catalytic effects, such as those mentioned in the substrates section, and inhibitory effects such as will be proposed later for Mg^{++} Provisionally, however we will assume that Ca^{++} is solely an activator—possibly just functioning in P O P hydrolysis thus in many experiments the solvent will be a solution of an inert electrolyte and an enzyme saturating concentration of a suitable calcium salt Of course work of other laboratories to be cited below will meet these conditions only roughly

Specific Sulfhydryl Reagents

In their classical work on SH enzymes Singer and Barron[19] early showed that myosin could be totally inhibited by parachlormercuribenzoate (PCMB) so it has been long presumed that the enzymatic site of myosin contains among others a sulfhydryl group More recently Kielley and Bradley[20] showed that besides this group at the site there existed in the molecule a second more reactive type of sulfhydryl group (probably not at the site) the masking of which accelerated the Ca^{++} saturated ATPase activity at neutral pH On this account a plot of activity vs number of SH groups titrated is diphasic as the more reactive groups are affected the activity is increased then as the less reactive groups react the activity falls (eventually to zero) Blum[21] has shown that other heavy metals besides mercury behave in the same way The purely "activation" response is best evoked by the class of reagents that form mixed disulfides with myosin when incubated at moderately high pH e g by S β aminoethyl isothiuronium (AET) Dinitrophenol behaves qualitatively like PCMB and AET and although it has never been clearly shown to be an SH reagent it probably belongs with this class of ATPase modifiers Kielley and Bradley[20] speculated that the most PCMB reactive SH groups in myosin might in some way chelate the inhibitory "intrinsic" Mg^{++} (see below) This speculation has been supported by the subsequent observation that AET treated myosin ATPase is less sensitive to Mg^{++} in

hibition and very recently by Tonomura s observation [3] that intrinsic Mg^{++} (not removable from myosin by ethylene diamine tetraacetate [EDTA] rinsing) is released by the enzyme on reaction with PCMB

Substances That Chelate Alkaline Earth Cations

Through the work of Tarver Friess [4] [5] and of Bowen [6] it was established that at neutral pH EDTA strongly accelerated the ATPase of myosin B dissolved in 0 60 M KCl and inhibited that of myosin B dissolved in 0 60 M KCl The result was no different if the enzyme was first rinsed with EDTA and then with KCl solution before adding the final test EDTA Recrystallized KCl was used throughout Since in the test system Mg^{++} (and only Mg^{++}) might have been expected to inhibit at 0 60 M KCl and activate at 0 60 M KCl it was speculated [5] that some form of inhibitory Mg^{++} bound to the enzyme so tightly as to defy EDTA rinsing was being masked by the test EDTA in the activation phenomenon * It is this Mg^{++} that Kielley and Bradley [0] thought might be held by SH groups on the myosin

Hydrogen Ions or pH

Although the dependence of myosin ATPase activity upon pH has been known for two decades its uninterpretable shape (at face value the curve shows optima around pH 6 4 and pH 9 5) has led to no useful inferences The attainment of high ionic strengths with minimal specific ion effects (by the use of TMAC) however has enabled some progress It has been shown [8] [9] that for AET treated myosin† the Ca^{++} saturated ATPase activity curve is like a titration curve with a pK between 6 5 and 7 0 and not very dependent on 1/T (very similar characteristics to creatine kinase and to the pH dependence of Zn^{++} inhibition of contraction of glycerinated muscle fiber [9]) The curve for untreated myosin ITPase has very much the same shape On the other hand as pointed out by Gilmour [31] the curve for untreated myosin ATPase (in which pains have been taken to avoid irreversible effects at high pH) looks like an ITPase curve in which some form of inhibition has been imposed at pH 7 8 It is cogent to recall here that Bowen s curves of EDTA activated myosin ATPase as a function of pH [6] also had a sigmoid (rather than bi optimal) aspect—rather like titration curves spread out over many pH units

Single Displacement Followed by Desorption

We now return to discuss what we have called the second major hypothesis about the myosin ATPase mechanism this is a hypothesis developed especially by Blum but with notable independent contributions by Koshland Levy and their associates and by Gilmour These authors suppose that while all substrates interact with myosin at the terminal pyrophosphate grouping those substrates (e g ATP CTP) with an NH group suitably placed on either the purine or pyrimidine ring suffer a strong additional interaction with the SH bound intrinsic Mg of the enzyme According to Blum this ring enzyme interaction retards the desorption of the diphosphate following P O P hydrolysis thus effecting an inhibition of what would be the activity in the absence of interaction Gilmour[31] has additionally assumed that this retarding interaction is pH sensitive and maximal at pH 7 8 To account for certain temperature effects Koshland and Gilmour separately have proposed that at some temperature between 20 and O C a conformational change in the enzyme draws away the SH Mg site from the substrate so that below the transition temperature the retarding interaction is no longer possible In contrast to ring NH substrates substrates such as ITP are assumed to be free of retarding interaction

Like all tentative hypotheses the foregoing

* This speculation is being disputed in current work from Tonomura s laboratory [23] since these workers find that after dislodging its intrinsic metals with PCMB the enzyme still responds to EDTA However a basic difficulty may lie in the use of KCl contaminated with Mg salts we have found[7] that in TMAC solution EDTA does not exert its characteristic effects For the while we feel the Mg hypothesis continues to have merit

† Similar results were obtained contemporaneously by J Blum using Cu treated myosin ATPase

leaves many facts unexplained (why is ITPase inhibited by small degrees of SH titration? Why would an NH Mg SH interaction respond to pH change around 7 8?) but it has many attractive consequences For example the hypothesis readily explains the pH curves why reagents as different as AET and EDTA cause similar effects and why Mg^{++} is not an inhibitor of ITPase activity Excluding mathematical booby traps (see Morales and Hotta[9] for discussion) the foregoing observations on pH dependence also point to an ionizable group at the active site with a pK of 6 5–7 0—possibly an imidazone or a phosphate of the enzyme substrate complex

Recently we performed experiments [7] whose results are encouragingly consistent with the general Blum Koshland Gilmour hypothesis We measured ATPase and ITPase activities as a function of pH in both ordinary and heavy water On the assumption that the isotope effect manifests itself primarily on the hydrolytic (P O P splitting) process we asked at each pH does D for H substitution make a difference in the observed rate? In the case of ITP the substitution always made a difference i e the rate in D_2O was always less than the rate in H O In the case of ATP the substitution made a difference at the pH extremes but at pH 7 or 8 it made no difference at all From our premise we concluded that the ITPase rate is probably always limited by the hydrolytic process and that this is also true of the ATPase rate at the pH extremes but that around pH 7 8 some other process is limiting the ATPase rate This other process would be the inhibitory ring enzyme interaction if so it should be eliminable by AET or by DNP treatment This prediction was confirmed with these treated enzymes the substitution did affect the ATPase rate even at pH 7 8 What the final fate of this ATPase theory will be depends on future research but its present status seems bright

Actin

Among the important properties of actin is its transformation from individual "monomer" units (M W ca 6×10^4 Gm) to more or less endless polymers i e, from its "G" to its "F" form upon such changes as the addition of salts or the reduction of the pH Straub and Laki and Bowen, discovered independently that in the G F transformation the ATP tightly bound to G actin was dephosphorylated in the course of the transformation Later, Mommaerts showed a precise 1 to 1 correspondence between the moles of orthophosphate released and the moles of G actin brought into polymer form The matter stood at this stage for several years except that during the interim Laki repeatedly emphasized the curious parallelism between conditions that govern myosin ATPase and those that govern actin polymerization and he raised the question of whether actin was an ATPase At the 1957 Tokyo Symposium on Muscle Chemistry this question was affirmatively answered by Oosawa Asakura and their associates at Nagoya University These workers showed that under suitable conditions (see below) an actin system could catalyze the *continuous* dephosphorylation of ATP, not merely the one shot amount that accompanied the conversion of almost pure G to almost pure F Before discussing actin ATPase mention must be made of another seemingly contradictory discovery by Oosawa s laboratory According to these workers the polymerization of actin is cooperative i e, if one commences with a very dilute solution of G actin and raises the G actin concentration a critical G actin concentration (dependent on $[Mg^{++}]$ pH temperature etc) is reached beyond which the further addition of G actin results in the progressive appearance of F actin whilst the concentration of G actin stays at its critical value Such behavior is typical of systems in which several of the condensing monomer units are *simultaneously* interacting in the formation of polymer According to the Nagoya workers an actin system exhibits maximal ATPase activity when G and F actins are co existing both in appreciable amounts (condition G—F) For a given value of total actin concentration the ratio C F will therefore vary with the state ($[Mg^{++}]$ temperature etc) and for a given state

the ratio will vary with the total actin concentration The claim is that whatever are the state and total concentration that achieve G→F that combination also maximizes the ATPase rate This elegant result is also a powerful indication that the ATPase is not arising from contaminations as was once seriously proposed Yet we have always felt that a fundamental difficulty might exist since if polymerization and dephosphorylation are rigidly linked then dephosphorylation as well as polymerization would be cooperative This circumstance would be quite unprecedented Each ATP being dephosphorylated by a myosin molecule for example is very remote and insensitive to the fate of fellow ATP molecules being dephosphorylated elsewhere thus far interacting enzymatic sites have existed only in the minds of theorists[3] It therefore seems to us very important that in their latest paper Asakura Kasai and Oosawa[33] provide evidence that actin ATPase is cooperative For example they find that upon the addition of salt the initial rate of splitting depends on a high power (3 5) of the G actin concentration and that the addition of F actin causes a burst of ATP splitting To provide a structural basis for the polymerization and ATPase effects Oosawa is now proposing a helical (rather than linear to account for interactive effects) possibly salt linked and rather delicate structure for F actin In support of this structure he has already shown acceleration of ATP splitting by 10 Kc sonic irradiation Apart from being contributions to the knowledge about ATPase the foregoing observations are opening the way for a possible intrinsic contractility in F actin a matter of critical concern to the model of contraction discussed in this symposium by Dr Podolsky

Although the enzymology of actin is still too sketchy to discuss at length mention should be made in closing of Strohman s[1] important new observations on actin ATP binding The absorption of Mg^{+} and Ca^{++} to actin monomers undoubtedly reduces repulsions and facilitates polymerization very possibly it also facilitates hydrolytic attack on I O P However Strohman now has evidence that such cations cooperate with SH groups of actin in the highly specific (ITP won t do) binding of ATP to the protein His observations are very reminiscent of the retarding interaction of ATP discussed above for myosin

Acknowledgment

The authors are very grateful to Drs Blum Brahms Hotta Oosawa Strohman and Tonomura for permission to quote from unpublished work

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Muscle Physiology and Contraction Theories

By Richard J Podolsky, Ph D

The structural basis of current contraction theories is the double array of thick and thin myofilaments revealed by the electron microscope The physiologic properties that characterize the contractile process are natural consequences of this structure if (a) during shortening the 2 sets of filaments move relative to each other and (b) the flux of chemical energy through the contractile mechanism is limited by interaction b tween complementary sites distributed along the _ sets of filaments Contraction models fitted to these ideas differ largely in the mechanism by which force is generated In the *sliding model* force is developed by mechanical interacti n between the thick and thin filaments and filament length remains constant during contraction In the *folding model* force is developed in the thin filament whic shortens during contraction Both mod ls quantitatively account for the force velocity relation and the Fenn effect They also accommodate the quick release phenomenon and predi t at least qualitatively the isotonic veloc ty tran ients that can be seen after qu k relea e f om tetanic ten on

BIOCHEMICAL PROCESSES in muscle cells have 2 exceptional characteristics The first of course is that they generate large forces The second which is not quite so obvious is that they proceed at a rate which depends on the motion of the cell Many properties of contracting muscle can be traced back to the influence of the motion of the cell on the chemical processes driving the con tractile mechanism an idea first proposed by Fenn[1]

It seems very natural that a muscle should lift a light load more rapidly than a heavier load However as Fenn wrote some years ago the more one tries to explain these sim ple facts the less obvious do they seem to be

The simple fact is shown in figure 1 the force velocity curve for the classical striated muscle the frog sartorius The circles are data from an experiment we shall discuss later The smooth curve is A V Hill s force velocity relation[2] The question is Why does the contractile force fall with the velocity?

In the early twenties the force velocity relation was explained with a viscoelastic model[4] along the following lines Upon acti vation muscle becomes an elastic filament like a spring The spring is immersed in a viscous fluid When the muscle shortens a given distance the available potential energy appears either as work or as heat The more quickly the muscle moves the greater the viscous force the greater the heat produc tion and the smaller will be the energy avail able for work Thus the fall in force with speed

The essential part of the viscoelastic model is that in shortening a given distance the available energy is independent of the me chanical conditions of contraction The corol lary that the amount of heat produced in a contraction increases with the speed was doubted as long ago as 1864[5] The unambig uous experiments of A V Hill confirmed these doubts

Figure 2 taken from Hill's classical 1938 paper[3] shows the critical experiment * Curve E is the total heat produced as a function of time when the frog sartorious is tetanized iso metrically Curves F G H and J show the heat liberated when the muscle is released at the time indicated by the first arrow and allowed to shorten a given distance Although the speed of contraction in J say is 5 times greater than in F the total extra heat due to shortening does not change Since it was clear that heat production did *not* increase with speed the viscoelastic theory was demolished

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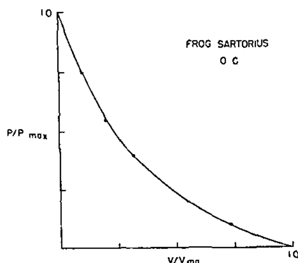


Figure 1

Relation between force P and velocity, V, in living muscle Experimental points are calculated from data of figure 12 smooth curve is the force velocity equation of A V Hill [3]

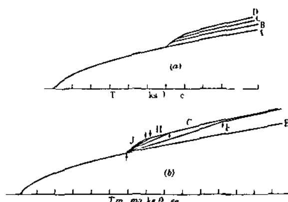


Figure 2

Heat production of living muscle Tetanically stimulated frog sartorius at 0 C (a) A isometric contraction B C D 1.2 sec after start of stimulus muscle is released and allowed to shorten various distances ($B < C < D$) under constant load (b) *E isometric contraction F G H, J muscle is allowed to shorten the same distance under various loads ($F > G > H > J$) (From Hill* [3]*)*

The error of course is that the energy available during contraction is not constant The *flux* of chemical energy into the muscle is somehow affected by the motion itself In other words some kind of mechanochemical control is built into the contractile mechanism The force velocity relation might be a reflection of this mechanochemical control if the tension generating process were continuously opposed or inactivated by the mechanical motion

This idea is schematized in figure 3 We assume that a sequence of chemical reactions drives the contractile mechanism One of these reactions M is closely linked to the contractile mechanism Since the chemical reactions are themselves linked like a train of gears the extent of the reaction during a contraction is limited by the turnover of M which in turn is some function of the shortening velocity The total energy produced during contraction depends on the extent of the reaction A + M This chemical energy* is partitioned between work and heat Conversely the rate of chemical reaction can be inferred from the rate of total energy production The trick in devising contraction theories is to make the link between mechanical and chemical processes such that both the total energy production and its partition into work and heat depend on mechanical motion in just the right way We shall first show what the ' right way is and then describe how several models manage to do this

Before leaving figure 3 I should like to mention that there is independent evidence from certain heat measurements that this representation of the sequence of events is close to the truth [6, 7] Perhaps the best justification though is that it accommodates the physiology of muscle very comfortably

Energy Production

How does the *rate* of chemical reaction change with the *rate* of mechanical contraction in living muscle? This can be inferred from the velocity dependence of the energy flux (fig 4)

Consider the heat first You will recall that Hill s heat measurements showed that the heat of shortening is independent of contraction speed (fig 2) This means that

* In this paper energy denotes the enthalpy of reaction $\Delta H = \Delta U + P\Delta V$ The energy available for mechanical work is the *free* energy $\Delta H - T\Delta S$ In these expressions U is internal energy P is pressure V is volume T is temperature and S is entropy

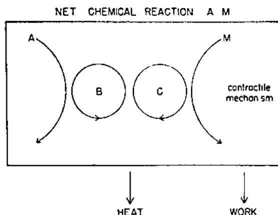


Figure 3

Scheme for chemical processes associated with muscular contraction

the *rate* of heat production must increase linearly with speed (fig 4 open region)

The rate of work production also depends on speed the dependence can be calculated from the force velocity relation When the muscle does not move (V = 0) it produces no work Also when it is unloaded (P = 0) no work is done This ties down the ends of the work flux curve (fig 4 shaded region) Adding the heat to the work we see that the total energy flux increases monotonically but not linearly with speed The rate of the rate limiting chemical reaction the one linked to the contractile mechanism must also increase with speed in exactly the same way To be acceptable a contraction theory should yield this relation naturally and quantitatively

Structural Basis of Contraction Theories

What mechanism regulates muscle chemistry according to speed? The double array of filaments revealed in Hugh Huxley s beautiful electron micrographs[8] provides a clue Figure 5 shows the characteristic thick and thin filaments * The thick filaments define the A band A second set of thinner filaments extend from the Z line through the I band into the A band there interdigitating with

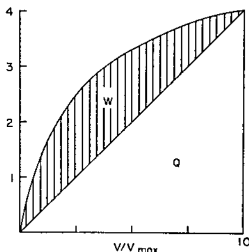


Figure 4

Relation between energy flux and velocity in living muscle Open region *rate of heat production* Shaded region *rate of work production*

the thick filaments The part of the structure that interests us is diagrammed at the top of figure 6

Andrew Huxley and Rolf Neidergerke demonstrated that when living muscle shortens the decrease in length takes place almost entirely in the light I bands the width of the dark A bands remaining constant [9] There are two obvious ways for this to come about the filaments could slide along each other or after anchoring its ends the thin filament could shorten by folding (fig 6) In both schemes only the I band shortens Also and this is important in what follows in both schemes there is *relative motion* between the 2 sets of filaments This means that if reactive sites were distributed along both the thick and the thin filaments and if some kind of interaction between these sites were stoichiometrically linked to the driving chemical reaction the relative motion of the myofilaments —and therefore the sites—would provide a natural basis for introducing *velocity* as a parameter in the chemical kinetics

Implications of Relative Motion Energy Production

The basic idea is shown in figure 7 Sites

* Figure 5 reproduced from H E Huxley J Biophys & Biochem Cytol 3 631 1957 By permission of the Journal of Biophysical and Biochemical Cytology



Figure 5

Double array of filaments in striated muscle Electron micrograph of longitudinal section of sarcomere (length = 2.5 microns) (From H F Huxley [8])

D are distributed along the thick filament and complementary sites K are distributed along the thin filament We assume that interaction *may* take place when these sites pass each other *If* interaction does take place a substrate molecule M is used up and the driving chemical reaction proceeds one step *

Proximity Time Is Rate Limiting

To understand the influence of velocity in the kinetics of such systems consider first a case in which the probability of interaction depends on the time K spends in the neighborhood of D *the shorter the time that is the higher the speed the lower the probability will be* The interrupted line in figure 8 shows how the probability decreases with speed if the K D interaction is first order in time Since at each interaction the driving chemical reaction proceeds 1 step the interaction probability in a transit of K past D will also be proportional to the energy released for a *given amount* of shortening The decrease in this quantity with velocity can be interpreted as the Fenn effect [1]

To calculate the number of interactions per unit of time we must remember that the number of chances a given K site will have to interact with D sites—the encounter rate—is proportional to the velocity (dotted line fig 8) The number of *successful* interactions will be the product of the probability and the encounter rate (solid line fig 8) Both this function and the rate of the driving chemical reaction in living muscle calculated from the energetics (fig 4) depend on velocity in the same way We conclude then that if complementary discrete sites were distributed along the 2 kinds of myofilaments the rela

* In the following a helpful mnemonic is to read D as Dragon K as Knight and M as Maiden

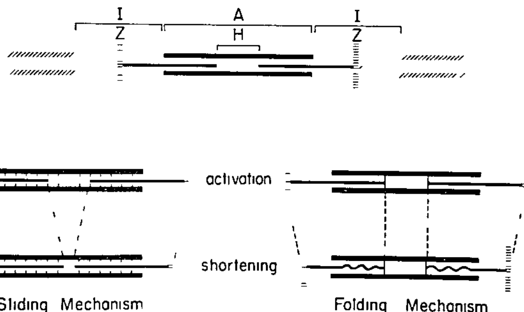


Figure 6

Hypothetical mechanisms for muscular contraction Upper Configuration of thick and thin filaments in resting muscle (after fig 5) Lower Change in filament shape and configuration in sliding and folding contraction models

tive velocity of the myofilaments could control the rate of interaction between these sites in exactly the same way as it controls the release of chemical energy in living muscle

Filling Time Is Rate Limiting

A physically different way of describing interaction between sites on moving filaments focuses on the time spent *between* rather than *at* encounters In this case we suppose that the substrate molecule M is carried by K past D If K is loaded with M an interaction takes place at D emptying K *regardless* of the speed The rate limiting step in this scheme is the filling of K with another M after it has been emptied by D The filling probability will be lower the shorter the time spent between D sites that is the higher the speed It turns out that if the law for filling empty sites is exponential in time which is not unreasonable the probability factor will have exactly the same form as in the previous case in which proximity time rather than filling time is rate limiting[10] This means that there will be no difference in the kinetics

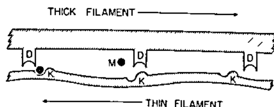


Figure 7

Distribution of sites along the thick and thin filament See text for explanation

of the 2 schemes for steady motions both explain equally well how motion controls the rate of energy release The different structure of the schemes will become important however when we consider velocity and force transients

The question of whether the thin filaments fold or slide during shortening has been side stepped This could be done because in *steady* motions the mathematics proves to be substantially the same for both cases

Force

What generates the contractile force? In a sliding model since the lengths of both the

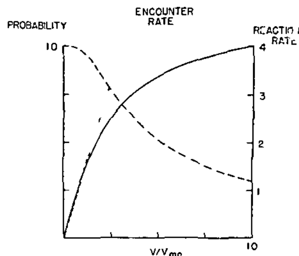


Figure 8

Chemical kinetics for reaction sites moving with relative velocity V. Interrupted line: *probability of interaction (rate limited by proximity time) or of filling (rate limited by filling time).* Dotted line: *encounter rate.* Solid line: *reaction rate = probability × encounter rate.*

thick and thin filaments remain the same, the contractile force must stem from mechanical interaction between the filaments. In a folding model, however, the contractile force is generated in the thin filament and its length is supposed to decrease during shortening.

A Sliding Model

The mechanical and chemical properties of a sliding mechanism in which the D site on the thick filament has the mechanochemical properties diagrammed in figure 9 was worked out by Andrew Huxley.[11]* D oscillates back and forth. When the K site passes, it can interact with D to form a mechanical connection. Since the probability of forming a connection in a D K transit depends on the relative speed, this is a special case of interaction in which proximity time is rate limiting. Each connection is in time broken. One step of the driving chemical reaction is associated with each connect-disconnect cycle.

If D and K connect, the elastic elements holding D to the thick filament will exert a force on the thin filament that is proportional to the distance of D from O. If the thin filament is moving to the left, connections with D to the right of O will make a positive contribution to the force. Conversely, if motion of the thin filament carries D to the left of O, a *force will be developed that retards the* motion.

To develop a net positive force—that is to ensure that there will be a greater number of pulling than retarding connections—Huxley postulates that D can connect to K only when it is to the right of O. The rate constant for breaking a connection also depends on x: it is small when D is to the right of O but large when it is to the left.

The force developed by the model depends on speed because the number of attachments and their distribution about the equilibrium position of the D site depends on speed. This is shown in figure 10, taken from Huxley's paper.[11] The ordinate is the fraction of D sites at a given displacement from the equilibrium position that are connected to K sites. When the filaments do not move, all the connections are on the pulling side of the equilibrium position; links can be made only on this side and there is no motion to carry them to the other side. In steady motion, the number of pulling links decreases, the retarding links tend to increase, and the force drops. At the maximum speed, the pulling and retarding forces balance and there is no net force. Huxley showed that, as the relative speed increases, these shifts in both number and distribution of links between filaments can account quanti*tatively for the force-velocity relation in living* muscle.

The diagram also shows why there *must* be steady motion for a less-than-maximum force to remain constant. Consider the distribution of connections when the speed, say, is one tenth of the maximum. If the motion should stop, after some time pulling links would form to the right of O, retarding links would open to the left of O, and the net force would increase. The original force could be re-established by relaxing the pulling links

*Figures 9 and 10 reproduced from A. F. Huxley: Progr. Biophysics 7: 255, 1957. By permission of Pergamon Press Inc.

or stretching the retarding links that is by displacing the connection contour to the left which of course is what happens when the thin filament slides past the thick filament. A steady force can be set up only when the motion in a given time interval just compensates for the net increase in pulling links formed in that same period

The diagram also explains what happens if tetanized muscle is quickly released Before release the links are all in the pulling position as shown in the distribution at the top of the figure If the muscle is moved so quickly that the links do not change their points of attachment, the tension will fall linearly as the links are carried past the equilibrium point and will vanish when the distribution becomes symmetrical about the origin Thus the model predicts that if muscle is moved very quickly the tension will drop to zero with a small displacement which is what actually happens[1, 13] This is a simple mechanical process and is due to the release of strain in the relatively short pulling connections between the thick and thin filaments

Notice that the distribution of links just after a quick displacement will be rectangular while the corners are rounded in the steady state Since for a given tension the motion depends on the shape of the connection contour this implies that the velocity just after a sudden drop in tension from the maximum to some intermediate value will not be the same as that after the steady state has been established There should be a velocity transient reflecting the transition of connection contour from a rectangle to the steady state shape We shall return to this point later

In summary in the contraction model analyzed by Huxley the 2 sets of myofilaments slide past each other The sliding motion arises from mechanical interactions between complementary sites The chemistry of the system reflects the mechanical motion since each mechanical interaction is coupled to a chemical reaction The model explains remarkably well the structural and energetic

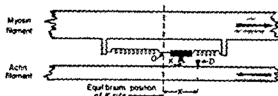


Figure 9

Mechanochemical element in sliding model of A F Huxley x is distance of thin (actin) filament site K from equilibrium position of thick (myosin) filament site D See text for further explanation (From A F Huxley[11])

changes that take place during contraction The least satisfactory element in it is the rather special nature of the hypothetical mechanochemical sidepieces on the thick filaments the pullers However it is not difficult for Nature to do things in ways which seem unduly complicated to physiologists "

A Folding Model

In a folding model the contractile force arises from a change in state of the thin filaments the thin filaments become elastic like a rubber band To make such a model work we must assume that force is generated when a substrate molecule M binds to (or reacts with) the thin filament at a K site and that the magnitude of the force is proportional to the number of occupied K sites[14] The force could arise from an electrostatic entropic process as has been eloquently argued by Morales and Botts[1] or from a polymer melting process as suggested by Pryor[16] and by Flory[17]

In an electrostatic entropic process a rubber like filament is stretched out by the mutual repulsion of distributed electrically charged groups At the equilibrium length the entropic force tending to shorten the filament is just balanced by the electrostatic force tending to extend it If some of the charged groups were neutralized by the binding of oppositely charged molecules the net electrostatic force would decrease and the filament would tend to shorten if length were kept constant force would be developed

In a polymer melting process the filament is initially extended by structural forces

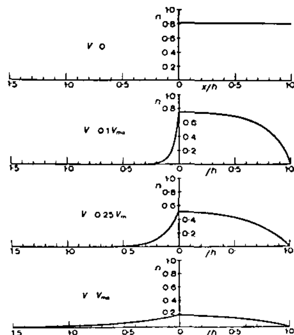


Figure 10

Distribution of links between thick and thin filaments in steady motion of sliding model of A F Huxley n is fraction of D sites at a distance x from the equilibrium position that are connected to K sites V is relative velocity h is maximum value of x (see fig 9) (From A F Huxley[11])

such as those that coordinate a crystalline solid When a critical temperature is reached the crystalline structure melts and the polymer becomes like rubber In this state if kept isometric the filament will exert considerable force The dotted line in fig 11 shows such a phase transition for an ideal crystalline polymer The melting temperature will be changed if substances bind to (or react with) the polymer the change will depend on the extent of the binding

Sharp phase transitions are characteristic of the ideal crystalline polymer Melting curves or real polymers are often gradual (solid line fig 11)[17] however in this case too the curve can be shifted by an amount that depends on the extent of binding Now if temperature is kept constant ($T = \tau$) and the extent of binding varied the extent of melting will vary If length is kept constant the force will increase with binding

Degree of melting in this process is analogous to charge neutralization in the electrostatic entropic process, both unlock the rubber like properties of the polymer The essential features are that (a) force is generated in a single filament which can shorten by folding and (b) force can change according to the number of small molecules bound to (or reacted with) the filament

A considerable amount of muscle physiology follows naturally from this hypothesis if we suppose further that there are sites on the thick filament that remove the force generating molecules from the thin filament by interacting chemically with them as they move by In other words, we suppose that there are interactions between the 2 sets of filaments in which filling time is rate limiting We have already shown that this scheme yields up the correct answers for the relation between rate of energy production and speed *

To *explain how the force velocity curve* comes out consider figure 7 again Suppose the force is a third of the maximum Then 1 binding (K) site out of 3 will be filled 2 out of 3 will be empty A substrate molecule from the environment will in time find its way to one of the empty sites When the site fills the force in the filament will rise above that of the load To re establish mechanical equilibrium the filament will shorten by folding until 1 of the filled sites passes a D site *which removes an M so that the force will* again be a third of the maximum The process will be repeated when another binding site is filled In the steady state with many sites in the game the rate of motion will be such that the emptying of *full* K sites passing D sites is just balanced by filling of *empty* K sites The force is the average occupancy of the K

* The energetics of steady motion does not depend on whether filling time' or proximity time is rate limiting However in the latter case (as was used in an earlier version of the folding model) it can be shown that the model does not accommodate the drop in force after quick release from tetanic tension On the other hand if filling time is rate limiting the folding model behaves properly upon quick release

sites the dependence of the average occupancy on velocity is the force velocity relation This turns out to be the same as that in living muscle

What happens when a muscle exerting maximum tension is suddenly released? (You will recall that in the sliding model because the 'pullers relaxed the tension dropped linearly with shortening) In this case if the motion is quick relative to the filling time full K sites will be rapidly emptied by reacting chemically with passing D sites and the tension will drop The tension will fall linearly with distance only until the force reaches half maximum Then because shortening is by folding rather than by sliding it can be shown that further motion will lead to a proportionately smaller drop in force which is what happens in living muscle[13]

As in the analogous situation in the sliding model just after a quick drop in force the distribution of filled sites along the filament is not the same as it will be somewhat later after the steady state is established This means that the isotonic velocity after quick release from tetanic tension will generally be different from the later steady velocity Some time must pass before the velocity settles down to the characteristic steady value

Mechanical Transients

We made a series of experiments to look for this transition phase[18] The study was made with tetanically stimulated frog sartorius at 0 C (fig 12)* The upper trace is *displacement* of one end of the muscle and the lower trace is *force* at the other end After full tension was developed the force on the muscle was suddenly lowered to and then maintained at a given value Each set of traces is for a different final value of the force The insert (fig 12*g*) is a control with the muscle replaced by a simple spring

Three regions are of interest To the left of the vertical line we see the quick release phenomenon The muscle is 35 mm long the tension drops to nearly zero when the end

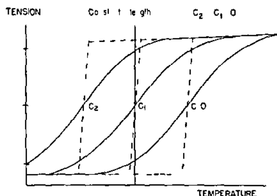


Figure 11

Tension development according to 'polymer melting' process Interrupted lines melting curves for *ideal crystalline polymers* solid lines *melting curves for real polymers The melting temperature decreases with the extent of binding (or reaction) of substrate with the polymer filament*

moves less than 2 per cent of this length (fig 12*f*) Depending on which model we favor this could be either relaxation of pulling links or emptying of binding sites Hill found a burst of heat during the quick release which he attributed to a high thermoelastic coefficient of what in the sliding model corresponds to the pulling spring[19] An alternative interpretation is that a chemical rather than a physical process is associated with the loss of tension as is the case in the folding model This interpretation also agrees with the studies of the insect physiologists who invoke ' in activation by release —as opposed to relaxation by release —to explain the very *high* frequency movements of certain insect muscles such as those driving the noisemaker of the locust[20]

At each force the velocity *ultimately* settles down to the characteristic steady value the lower the force the more rapid the motion (The force velocity curve in figure 1 was drawn from these data) The remarkable linearity of the displacement traces supports the idea that the motion is controlled by a feedback mechanism

The interesting findings in these experiments are the variations in speed (to the right of the vertical line) before the velocity settles down to the steady value there is a

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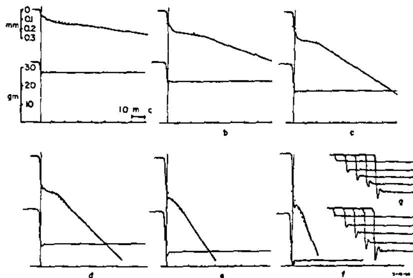


Figure 12

Response of muscle to a sudden change in force Upper trace displacement, lower trace tension, frog sartorius tandard length = 35 mm 0 C Muscle is initially at the standard length and the record is started after full tetanic force P_{max} has developed P/P_{mo} (a) 0 84 (b) 0 69 (c) 0 55 (d) 0 40 (e) 0.26 (f) 0 10 In (g) the muscle is replaced by a simple spring (From P dolsky[18])

characteristic *isotonic* velocity transient for each tension step One contribution to these transients is the variation in velocity corresponding to the setting up of the steady state of motion in the contraction models we have discussed However, since the experiments were made with the whole sartorius muscle there is also a contribution due to the interaction of muscle fibers of different lengths To sort out these 2 components of the transient seen in the intact muscle we are repeating these experiments with preparations containing only a few fibers this should reduce the contribution of fiber interaction We are also calculating the transients predicted by the sliding and folding contraction models to see which model accommodates the experimental data better* In these studies attention is focused on the *approach* to the steady state of motion rather than on the steady state *per se* then the contraction kinetics of the 2 models can be distinguished

Conclusion

In summing up I should like to point out that the contraction theories work because of 2 basic assumptions The first—and this can really be elevated to the status of fact rather than assumption—is that the 2 sets of myofilaments move relative to each other in shortening The second is that the flux of chemical energy through the contractile mechanism is limited by interactions between complementary sites distributed along the 2 sets of filaments

Two models were fitted to these ideas They differ largely in the mechanism by which force is generated In the *sliding* model force is developed by mechanical interaction between filaments and there is no change in filament length during shortening In the *folding* model force is developed in a single filament which shortens during contraction In both models there is interaction between the mechanical motion and the force generating mechanism Chemical processes tend to increase the mechanical force in the sliding model pulling connections are made in the folding model binding sites which generate force if filled become filled If the load is constant these processes tend to create a mechanical imbalance which however can be righted by shortening in the sliding model pulling connections become weaker in the folding model binding sites are emptied Chemical and mechanical equilibrium are incompatible for forces less than full tension However since shortening tends to inactivate the force generator a less than maximum force can be maintained if there is steady motion Conversely if the load is less than

* This study is being made in collaboration with Dr N Z Shapiro of the National Institutes of Health

the maximum force that can be generated there *will* be steady shortening This is the force velocity relation

Comparing the models with living muscle both of them quantitatively account for the changes in force and energy flux with velocity The quick release phenomenon is also accommodated mechanically in the sliding model and chemically in the folding model Both predict at least qualitatively the isotonic velocity transients that can be seen after quick release from tetanic tension

This list of accomplishments suggests that some of the devices used to get them might actually be built into living muscle

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The Regulation of Metabolism and Energy Release in Contracting Muscle

By WILFRIED F H M MOMMAERTS PH D

A brief review has been given of our present knowledge of the nature of the chemical events coupled with muscular contraction Although there are experiments that fail to demonstrate a breakdown of ATP in the contraction of living muscle these may yet yield to special explanations leaving the conclusion that a breakdown of ATP is the reaction most closely related to the mechanical activity of the contractile structure Owing to a rapid rephosphorylation reaction this event appears in the form of a breakdown of phosphocreatine The amount of energy liberated in contraction may be written as the sum of 3 quantities (a) activation energy (b) shortening energy and (c) work It appears that chemically these correspond to 3 distinct quantities of phosphocreatine breakdown The matter is complicated by the fact that the activation energy is not constant but may diminish as the muscle shortens This may be little noticeable in frog sartorius muscle (dependent on the treatment) but appears to be pronounced in the heart where it leads to the classical observation that work against a high pressure is performed less efficiently than work raised by ejections of an increased volume

IN MORE THAN one respect this opportunity to discuss the biochemical origin of muscle energy and the mechanism of the regulation of its release comes at an inconveniently early moment I would have preferred to postpone it until after the completion of some of our current lines of experimental work However there is no certainty that this work will be successful within the foreseeable future and it may be of interest to the audience to discuss the present status of the question and to present our preliminary views and the remaining formidable problems as we now see them

Most of our knowledge about the problem at issue has been obtained through the myothermal studies of A V Hill and it may be well to give a brief outline of these first The significance of such measurements is that they give a complete accounting of the total energy that is mobilized as far as this is not turned into external work the latter can be measured mechanically—or also thermally if at the end of the contraction the work is returned to the muscle (i e if the lifted load falls back while stretching the muscle) and so warms it up by an amount equal to the work done originally * Furthermore the myothermic method also allows us to establish the time course of energy liberation to a degree with which biochemists may eventually hope to catch up but certainly have not done so yet Thus it has been established that in a strictly isometric contraction a definite amount of energy is liberated during the contraction phase of the cycle this energy is called the activation heat A, or alternatively the maintenance heat especially in the case of a tetanus when one emphasizes the maintenance rather than the initial establishment of the active state

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* That the work done upon a relaxing muscle appears quantitatively as heat is not as self evident as it might seem One expects this when work is dissipated by friction as in one of the Joule experiments but not e g when the lead is hung on a steel wire which cools when so stretched reversibly because of the preponderance of the energy term $\delta U / \delta_L$ in the equation

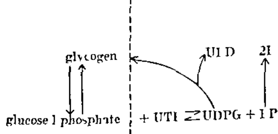
$$\left(\frac{\delta W}{\delta}\right)_T = \left(\frac{\delta U}{\delta}\right)_T + T\left(\frac{\delta S}{\delta_l}\right)$$

In the case of relaxing muscle the equality would indicate either that the dissipation of work in relaxation is completely irreversible or that in this equation the entropy term predominates i e that the relaxing muscle is a fairly perfect rubber

Again we have the fact that when the muscle shortens there is a liberation of an additional amount of heat strictly proportional to the shortening ΔL which is called the shortening heat. Finally there is the fact that any work that is being done is in addition to these two heat quantities: a muscle lifting a load will first develop the activation and shortening heat corresponding to the distance shortened and the work $P \times \Delta L$ is in excess of this, appearing as heat when in relaxation the muscle is stretched by the falling load. Thus we can group the energies into two categories: the overhead needed for the activity of the mechanism and the actual work added to this without further heat effects. These statements which we owe primarily to the classical papers by Fenn[1] and Hill[3,4] can be summed up as

$$\Sigma(\Delta H) = A + a \cdot \Delta L + W \quad (1)$$

In relaxation, apart from any external work being returned to the muscle, there is no measurable heat production or uptake; this lack is commonly regarded as evidence that relaxation is energetically passive and not coupled with chemical reactions. Such a view requires some elaboration because (apart from the fact that the thermoneutrality of relaxation may be due to a coincidental cancellation of the heat effects of several processes which we cannot affirm or deny) if the activation of the working substance is exothermic, why is not the opposite process endothermic? A comparison may make this clearer. Let us consider the cyclical synthesis and breakdown of glycogen pretending with relatively small error that the reversible transformation glycogen ⇄ glucose-1-phosphate is thermoneutral.



Here the reactions on the left side are reversible although in practice under actual circumstances in muscle it would proceed in the direction of glucose-1-phosphate formation (which in our analogy will be compared with the contractile material at rest). To make glycogen (here representing active muscle) we have to couple the process with the exothermic breakdown of UTP, in analogy with the activation of muscle by the breakdown of ATP (see below). Thus we can have a cycle in which glycogen synthesis (activation of muscle) is exothermic, glycogen breakdown (relaxation) about thermoneutral. The analogy breaks down of course among other things because the active muscle can perform work and when doing so consumes more of the associated exothermic reaction. It suffices to illustrate the main point, however: activation is coupled with an exothermic biochemical process, relaxation is not, to our knowledge, coupled with anything. In more general terms let us consider an energy yielding reaction such as ATP breakdown with its heat effect ΔH, coupled with a transformation A→B in activity while the reversal B→A takes place in relaxation without associated chemical reaction. Now the laws of thermochemistry require that the total ΔH of the energy supplying reaction appear at the end (apart from unreversed external work) regardless of the specific nature of the A→B→A processes. But if A→B and its reversal have themselves a finite heat effect this would add itself to that of the coupled reaction in contraction and appear with the reversed sign in relaxation. Hence unless we are misled by a coincidence, the observations indicate that the transformations within the working substance, while coupled with an extraneous exothermic reaction, are approximately thermoneutral.

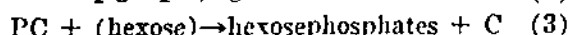
Since this conclusion will need considerable scrutiny we shall not attempt to explore its full scope. It will merely be used to introduce the following problem: if the energy for each of the manifestations of muscular activity is derived from a coupled reaction, it would be important to establish a

complete biochemical energy balance for these events Specifically is it possible to assign definite amounts of chemical change with each of the three entitles A, a ΔL and W and are these entities associated with different reactions, or with separate quantitie of the same reaction?

Before proceeding further however we must first decide what reactions are available In previous periods there has been much discussion about inogen lactic acid phosphorylcreatine and—more recently—K Na exchange and direct oxidative energization but more and more compelling if not entirely direct evidence points toward the role of ATP as the primary energy donor The foundation of the ATP theory is very strong indeed (as I have expressed elsewhere[5]) it differs from all previous theories by the fact that it has a foundation Yet an assumption that is so basic requires direct experimental proof and here we find ourselves in a peculiar state of uncertainty Some years ago Fleckenstein et al and Davies[6,7] and I[8,9] showed that after a rapidly interrupted contraction it was not possible to detect a diminution of ATP or of phosphorylcreatine (PC) which might have reversed a primary ATP breakdown We should not regard this evidence as proof that the ATP theory is false On the one hand Carlson and Siger[10] have recently implied that our early experiments were simply in error although I do believe that they were well performed I also feel that a good deal more work is needed to elucidate the various aspects of the problem On the other hand there is a good possibility that in such experiments a primary breakdown of ATP may be concealed by a minor amount of another phosphoryl donor We (as well as Davies et al[11]) are now able to detect consistently a liberation of inorganic phosphate early in contraction of frog sartorius muscles In our current experiments this release of energy is correlated with an equivalent formation of creatine (in complete agreement with the ATP PC theory) but in an earlier recent series the PC breakdown was not demonstrated and there was often a diminution of a highly labile phosphate compound XP Moreover in our current series XP is present but does not change in the immediately preceding series XP was absent It seems best to defer judgment until more experimental work has been done Meanwhile I should like to maintain as did Hill[1] in 1950 that a concept of such paramount significance cannot be accepted as final until it is based upon direct proof

It is clear on the other hand that during more prolonged activity e g in a series of twitches or of brief tetani a breakdown of PC does occur This breakdown of PC presumably reflects a primary breakdown of ATP although strictly speaking this relationship has not yet been shown to apply to living muscle since the ATP does not decrease until after a sizeable decrease in PC Without belittling the primary role of ATP we shall present our work in terms of PC breakdown because that is what we measure and because it is this breakdown that contributes to PC reaction heat It is best demonstrated with iodoacetate poisoned muscles studied anaerobically because in such muscles there is no possibility of a resynthesis of PC The following reactions may then occur


$$PC \rightarrow P + C \quad (2)$$


$$PC + (\text{hexose}) \rightarrow \text{hexosephosphates} + C \quad (3)$$

Dependent upon the circumstances reaction (2) or (3) may predominate Work at low temperature suppresses (3) and this is advantageous because once phosphorylation of glycogen occurs with the P formed in reaction (2) there may also be further phosphorylation of fructose 6 phosphate at the expense of more PC the latter reaction is not connected with mechanochemical activity and therefore contributes an error to the measurement of the PC that is broken down and mechanically utilized Reaction (3) is not always fully suppressed at 0 C in an extensive series in which it was not suppressed we could establish that its occurrence is not correlated with shortening and work In other experiments that showed only re

action (3), shortening and work went on just as well. This leads to the first conclusion: variations in energy produced in the three categories A, a ΔL and W are not derived from different reactions but all result from the breakdown of PC according to reaction (2). The question is then: can we detect various parcels of PC breakdown that correspond quantitatively with A, a ΔL and W?

We have devoted a great deal of time to the following experiments: of each pair of muscles one served as the resting control, the other performed, e.g., 12 tetanic contractions against a certain load, the shortening ΔL was measured, and the PC breakdown determined from the difference in composition. A number of such results were then plotted as chemical change per gram per contraction against the shortening per length. If the results would conform to equation (1), a plot as in figure 1, curve A, would result. The actual findings displayed a dismaying amount of scatter, and their graphic representation supplied a perfect illustration of Fisher's[13] dictum: "Diagrams prove nothing, but bring outstanding features readily to the eye; they are therefore no substitute for such critical tests as may be applied to the data, but are valuable in suggesting such tests and in explaining the conclusions founded upon them." Such critically designed experiments are now in progress, but at this early stage we shall have to see what we can do with the older data.

One thing is quite clear at the present stage: muscles contracting against a moderate load and so doing about optimal isotonic work decompose more PC than do muscles that are not performing work. Hence the biochemical counterpart of the "Fenn effect," the factor W in equation (1), seems to have been demonstrated, although I would postpone a quantitative discussion until the termination of properly designed and evaluated paired experiments. Also we find that at zero shortening the activation metabolism A is of the order of 0.3 micromoles per gram per contraction, in good agreement with our

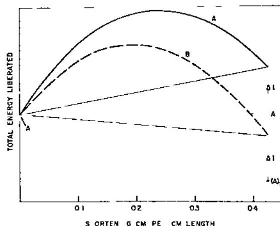


Figure 1

Diagram of the possible dependence of the total energy liberation upon the shortening of stimulated muscle as determined by the isotonic load. In curve A the energy at any given degree of shortening consists, as in formula (1), of the activation heat A_f, the shortening heat a ΔL and the work W. In curve B a ΔL and W appear as before, but the activation heat $(A)_T$ is variable and diminishes with the resulting length of the muscle.

original estimates[14] and with other recent experiments.[10] But as to the factor a ΔL the situation is less satisfactory. Not only was the variation of the points too great to estimate the actual course of the curve, but it appeared that in the region of maximal shortening the scatter exceeded the limits that could reasonably be expected from the variability between two muscles of a pair. Indeed direct comparisons between maximal and small shortening (with roughly the same amount of work) even showed that the shortening muscles in certain series decomposed less creatine than the nonshortening ones, contrary to expectation and contrary to the results of larger experimental series.*

While further direct experimentation along these lines will establish the direct biochemical evidence, some insight already exists into the reason for the variable results. First,

*This unexpected result, on the other hand, seems to have been the rule in the parallel work by F. D. Carlson of Johns Hopkins University. Neither his nor our work has been published, but both have mentioned these matters at several scientific meetings.

in equation (1) we assumed that A is a constant. However we know from Aubert's work[1] that this is not the case: in addition to being determined by a time factor that we have assumed to be constant, A depends on the length or primarily upon the tension of the muscle,* and while over certain ranges (such as those preferably employed in Hill's 1938 study[3]) the variation of A is small, A is in general much less in a muscle that is shortened to well below the natural length. Therefore we must write

$$\Sigma(\Delta H) = (A)_T + a \quad \Delta L + W \qquad (4)$$

Curve A in figure 1 can then only occur if in a special situation A is constant. If, on the other hand, the change in A upon shortening is considerable (or if a is less than usual), a maximally shortening muscle may well produce less heat and engage in less metabolism than an isometrically contracting one:

$$A_I > (A)_T + a \quad \Delta L \qquad (5)$$

In such case we would obtain curve B of figure 1. The literature is not explicit in this respect. According to Hill[3] A does not seem to vary greatly in the experiments on which the concepts were derived (but see Hill[3] pp. 169-170), but recent calorimetric results by Tigyi[16] and a sleight of hand extrapolation one can make of some of Fenn's[1] curves as well as the data of Nachmansohn (Tables X and XI[17]) are in accordance with equation (5). Our own exploratory results show that the quantitative relationships may differ from muscle to muscle, although more instances so far have been in the direction of curve A than of curve B. Clearly, there can be any number of intermediate cases between the extremes of equations (1) and (5). Therefore it is likely that our erratic results and those with the opposite tendency of Carlson are in full agreement with equation (4), meaning that each of the terms, activation, shortening and work energy, are associated with a definite quantity of the same overall reaction PC→P + C. But the accurate establishment of these relationships (including the parallel but not necessarily symmetrical case of negative work) will still require a great deal of painstaking work.

Believing that there should be a balance between experimental and theoretical work, I am not tempted at this moment to speculate on the mechanisms by which the muscle determines how much biochemistry to call upon during a given act of activity. There are many facets to this. On the one hand, what causes metabolism to be so greatly intensified? It is not entirely a matter of its being irrevocably coupled with the mechanical change that is elicited by stimulation, because it is known that metabolism can be greatly accelerated without a corresponding increase in mechanical activity by raising the external K concentration (an effect that requires the presence of Ca, just as excitation-contraction coupling does[18]). On the other hand, what determines the quantity of energy that is mobilized? That the activation energy depends on length and that a shortening energy does occur are not beyond imagination, since changes in length do involve a change in some configuration. Although it is harder to visualize the mobilization of extra energy for work, even the mystery surrounding this problem might disappear if it were formulated in different terms, e.g., relating the rate of energy production to the velocity and extent of shortening.

Finally, it may be stated that, while the present uncertainties, not only of the experi-

* In making this distinction between length or tension as the determinant variable, I am not alluding to the inquiry whether muscle is primarily a tension or a shortening generator, since I do not know if this is a valid question. The distinction appears when we try to estimate the maintenance heat of a muscle during shortening or stretch. Should one study the change in length from point to point and take the corresponding value of A from an empirically determined plot of A against l? Or should one take the A associated with the prevailing tension (which in turn depends on the velocity H)? In Aubert's experiment A depends linearly on the tension; this relationship is one of the reasons why we tend to favor the latter decision. Whatever the final decision, it is clear that with respect to the maintenance of heat, the distinction assures a real operational meaning.

mental results but even of the theoretical expectations may be disturbing it has its tranquilizing aspects as well A good deal of thought has probably been given lately to the problem of harmonizing certain properties of the myocardium (e g the lower efficiency when working against high pressure i e at lesser shortening than when the same work is done by ejecting a large volume of blood against a low resistance) with the Hill laws formulated in equation (1) these efforts have largely remained unpublished probably because they were unsuccesful The independent variability of the parameters of equation (4 enable us to treat such different cases by the same approach this independence also suggests that the separate study of A as a function of the conditions, and of a and W in conjunction with other dynamic quantities and with their metabolic correlates will be a rich field for future investigation

So viewed the present status of the field is not really disappointing it is merely that we have looked upon the matter from too simple a viewpoint The inability to obtain prompt answers has at least caused the problems to be formulated more realistically

Acknowledgment

The biochemical investigations alluded to in this presentation have been carried out during the last four years in cooperation with K S rIydarian and A Wallner M O Schilling has m an h le de el ped our instrumentation along the lines of A V Hill s methodology for the measurement of heat production in muscle During the few months since our explorations in myothermic work have been carried out by Dr B C Abbott (Department of Zoology The University of California Los Angeles) and Profes or S L Dart (Department of Physics Claremont College for Men Claremont California on leave in our laboratory)

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High Energy Phosphates and the Force of Contraction of Cardiac Muscle

By Robert F Furchgott Ph D and Kwang Soo Lee, M D Ph D

This paper reviews studies performed by the authors and by others on the levels of high energy phosphate compounds—adenosine triphosphate (ATP) adenosine diphosphate (ADP) and creatine phosphate (CP)—of cardiac muscle under various conditions influencing contractile force Interference with energy metabolism decreases both contractile force and high energy phosphates especially CP Certain types of experimental ' failure are associated with decreases in high energy phosphates however other types of ' failure occur without significant decreases in these phosphates In addition marked decreases or increases in contractile force independent of significant changes in high energy phosphates can be produced by drugs by changes in heart rate or by alterations in extracellular concentrations of cations A decrease in force when levels of high energy phosphate are normal may be attributed to a deficiency in the utilization of these energy stores for mechanical work The nature of this deficiency has been analyzed in isolated cat papillary muscles by simultaneously determining the activity oxygen consumption and contractile force per beat In the case of decreases in contractile force due either to reduced heart rate or to spontaneous heart failure the deficiency may be attributed almost completely to a loss in efficiency in the conversion of chemical energy in the high energy phosphates to mechanical energy (work) Cardiac glycosides in restoring contractile force do so by restoring the efficiency of this conversion In the case of decreases in contractile force resulting from lowered extracellular Ca the deficiency may be attributed partly to a loss of efficiency in this conversion and partly to a reduction in amount of high energy phosphate utilized per beat

IT IS NOW generally agreed that the energy for muscle contraction comes either directly or indirectly from the splitting of high energy phosphate bonds Although there may be other compounds that account for a small percentage of the total high energy phosphate bonds of cardiac muscle it is *reasonably certain that 90* per cent or more of such bonds occur in adenosine triphosphate (ATP) and creatine phosphate (CP)[1] In cardiac muscle under steady state conditions it may be assumed that the rate of utilization of high energy phosphate bonds for mechanical work and for other processes in which free energy is required is balanced by the rate of resynthesis of high energy phosphate bonds as a result of the coupling of phosphorylation with metabolism of food stuffs primarily oxidative metabolism If the energy for contraction is derived from high energy phosphate bonds then it might be expected that some correlation could be found between the level of the principal high energy phosphate compounds in heart muscle namely ATP and CP and the strength of contraction *The first part of this paper* will deal with the results of experiments carried out in the laboratories of the authors as well as in other laboratories to investigate the relationship between high energy phosphate content and the contractile *strength of cardiac muscle* The inescapable conclusion to be drawn from these results is that under many conditions which markedly alter the contractile strength of heart muscle there is no corresponding alteration in the levels of high energy phosphate compounds These results have led us[3,4] to postulate as Wollenberger[-] did previously that decreases in strength of cardiac contraction under many experimental conditions are not due

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Table 1

Concentrations of Inorganic Phosphate Creatine Phosphate and Adenine Nucleotides in Mammalian Cardiac Muscles

C d t a ly ed	C t tl i µM/Gm. t					R f
	IP	CP	ATP	ADP	AMP	
Dog left ventricle (apex) in situ	1 9	1[illegible]8	5 7	—	—	Wollenberger et al 1960
Dog left ventricle of HLP	5 6†	7 6 †	4 3	—	—	Fawaz and Tutunji 1957[15]
Cat ventricle (apex) in situ	5 †	[illegible] †	3 39	0 [illegible]9	0 19	Fleckenstein et al 1959
Cat papillary muscle in vitro	3	7 04	[illegible] 81	0	0 17	Lee et al 1961
[illegible]at ventricle in situ	3 8	[illegible] 8	4 12	0 71	0	Fleckenstein et al 1958
Rabbit left ventricle in situ	1 9	8	—	—	—	Wollenberger et al 1960
Guinea pig left ventricle in situ	0	10 9	—	—	—	Wollenberger et al 1960
Guinea pig left ventricle in situ	1	8 38	5 59	0 64	0 34	Feinstein 1960
Guinea pig left ventricle in situ	1 [illegible]	0	3 48	1 0	—	Hochrein and Doring 1958
Guinea pig left ventricle HLP	8	01	3 1[illegible]	0 88	—	Hochrein and Doring 1958
Guinea pig left atrium in vitro 3	6 4	4 19	41	0 43	0 [illegible]3	Furchgott and de Gubareff 19 8
Rabbit atria in vitro 30	4 3	3 9	1	0 8	0 31	Fleckenstein et al 1959

Estimated from acid labile phosphate assuming 90% arises from terminal phosphates of ATP
†Figures probably reflect some conversion f CP to IP during freezing procedure

to a deficiency of high energy phosphate stores but to an impairment or deficiency in the utilization of such stores for contraction

Deficiency in utilization of high energy phosphates for contraction may result from a lower rate of utilization per contraction at essentially normal efficiency for conversion of phosphate bond energy into mechanical energy an essentially normal rate of utilization per contraction at decreased efficiency or a combination of these 2 conditions The last section of this paper will be directed at the problem of the nature of the deficiency in the utilization of chemical energy for contraction under certain experimental conditions that lead to marked alterations in strength of contraction with small or insignificant changes in high energy phosphate content

Levels of High Energy Phosphate Compounds Under Control Conditions

Over the past 10 years the reported levels for high energy phosphate compounds in cardiac muscle especially that of CP have risen considerably This is in large part due to the development of more refined procedures for the determination of these compounds—with better methods being applied not only in the analyses of extracts of cardiac muscle but also in the quick freezing of the tissues and in the extraction of powders made from the frozen tissues[1 3 6-9] Table 1 presents data obtained by ourselves and a number of other investigators with procedures that we feel give reasonably accurate values for the steady state levels of CP and ATP as well as of inorganic phosphate (IP) adenosine diphosphate (ADP) and adenosine monophosphate (AMP) Some of these values apply to hearts in situ some to heart lung preparations (HLP) and some to isolated beating cardiac preparations in vitro All of the preparations were under control conditions in the sense that contractile strength was at a study level and there was no indication of 'failure'

Table 1 shows that ventricular muscle CP ranges from about 7 to 12 micromoles/gram and ATP from about 3 5 to 5 5 micromoles/gram The levels of ADP are much lower than those of ATP ranging from about one fourth to one twentieth of the latter in the different preparations The differences in the levels of both ATP and CP reported by different authors probably are due in part to the use of somewhat different procedures and in part to actual differences in levels among species It would appear that in heart lung preparations and in isolated papillary muscle in good physiologic condition the

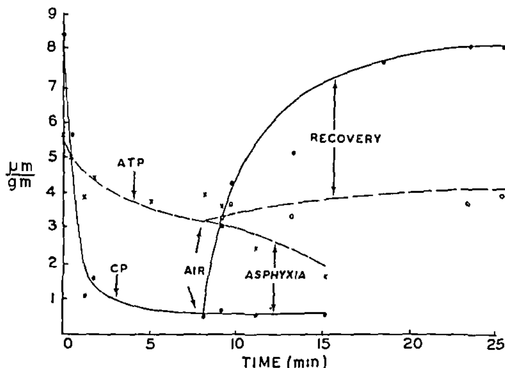


Figure 1

The effect of acute asphyxia and subsequent recovery from asphyxia on creatine phosphates and ATP in guinea pig heart in situ Each pair of points at any given time represents levels in a single animal except for pair of points at zero time which represents mean levels in control animals Asphyxia was started at zero time Continuous falling curves are fitted to points obtained with 8 animals subjected to varying periods of asphyxia Rising curves are fitted to points obtained with 6 animals after varying periods of recovery after resumption of artificial respiration (air) after 8 minutes of asphyxia (Data of Feinstein[7] used with his permission)

levels of CP and ATP are essentially the same as those found in the heart in situ[9-11] in any given species

The ATP and CP levels in atria (table 1) are considerably lower than those in ventricles in the same species For example in our laboratory we have found that isolated atria of guinea pigs contain only about half as much CP and ATP as do ventricles in situ[3 7] This smaller content of high energy phosphate compounds in atrial muscle is not too surprising in view of the findings of earlier workers that the acid labile phosphate (mainly from ATP) of atrial muscle is only about one half of that of ventricular muscle in the rabbit and dog[1 14]

Influence of Inhibition of Oxidative Metabolism

Conditions or agents that interfere with oxidative metabolism would be expected to lower the level of high energy phosphate in cardiac muscle if the rate of utilization of these compounds for contraction and other processes requiring free energy exceeded the rate of their resynthesis by the inhibited metabolism It is therefore not surprising that the high energy phosphate levels fall markedly below control levels when cardiac muscle is subjected to a lack of oxygen or to agents that inhibit oxidative metabolism as shown in table 2 In all cases in which metabolism is impaired there is also a very marked reduction in contractile strength

Note that in every case the fall of CP is greater than that of ATP and that in some cases a marked fall in CP is accompanied by only a small or insignificant fall in ATP The fact that CP is much more sensitive to impairment of metabolism than is ATP was

Table 2

Changes in High Energy Phosphates Accompanying Decreases in Contractile Strength Produced by Impairment of Energy Metabolism

Condition or agent impairing energy metabolism	Type of preparation	% Change from control level: Contractile strength*	CP	ATP	Reference
Anoxia for 18 min	Guinea pig atrium in vitro	−93	−69	− 7	Furchgott and de Gubareff 1958[3]
Anoxia for 30 min	Cat papillary muscle in vitro	−78	−77	−51	Lee et al 1961[20]
Asphyxia for 8 min	Guinea pig ventricle in situ	>−50	−94	−3	Feinstein 1960
Hypoxia (4% O₂) for 30 min	Rat ventricle in situ	Marked fall	−70	− 1	Fleckenstein et al 1959
Dinitrophenol	Dog ventricle HLP	−6	−63	0†	Fawaz and Tutunji 1957[19]
Fluoroacetate 15 mg	Dog ventricle HLP	−78	−59	−1 †	Fawaz 1956[21]
Phenylbutazone	Guinea pig ventricle HLP	Marked fall	−50	−35	Hochrein and Doring 1958

*Indices of contractile strength contractile amplitude for guinea pig atrium contractile force for papillary muscle maximal isometric pressure for guinea pig ventricle in situ cardiac output for heart lung preparations

†Estimated from acid labile phosphate assuming 90% arises from terminal phosphates of ATP

also indicated in earlier work on the effect of anoxia and metabolic inhibitors on the labile phosphates of cardiac muscle[1, 15–17]

From the results in table 2 it would appear that under adverse metabolic conditions decreases in the strength of contraction are more closely related to decreases in CP than to decreases in ATP In this connection the results of some experiments by Maurice Feinstein[7] obtained while he was a graduate student in our department are worth citing In these experiments he produced asphyxia in open chest guinea pigs by tracheal occlusion for varying periods In some of the animals he reinstituted artificial respiration after 8 minutes of asphyxia in order to follow the process of recovery Figure 1 shows his findings on the levels of ATP and CP at various times after the onset of asphyxia and also at various times during the recovery from asphyxia The fall in CP was much more rapid and extreme than the fall in ATP Moreover after reinstitution of artificial respiration CP returned to essentially the control level within about 10 minutes whereas ATP showed only a slight recovery over a period of almost 20 minutes In most of the animals used the left intraventricular pulse pressure was continuously followed over the course of the experiment and in some animals the maximal isometric pressure that the left ventricle could produce was determined at intervals by temporary complete constriction of the ascending aorta Using ' maximal isometric pressure as an index of contractile strength it appeared that contractile strength was close to normal as long as the level of CP was at least 20 to 30 per cent of the control level but that decreases of CP to still lower levels were associated with marked decreases in contractile strength In the recovery period after reinstitution of artificial respiration contractile strength was essentially back to normal by the time (less than 1 minute) CP had been restored to about 30 per cent of the control level even though there was no increase in ATP above its depressed level within the same period

From his experiments on cardiac asphyxia in situ Feinstein concluded that there was no correlation between decreases in contractile strength and decreases in ATP content but a fairly good correlation between decrease in strength and extreme decrease in CP content Hochrein and Doring[9] on the basis of their results with guinea pig heart

Table 3

Changes in High Energy Phosphates Accompanying Changes in Contractile Strength Produced by Drugs or Alterations of Experimental Conditions

Drug or experiment l condition	Type of preparation	% Change from control level		
		Contra strength	CP	ATP
High Ca in medium	G P atrium	+310	0	0
	Cat pap m	+160	0	−25‡
Low K in medium	G P atrium	+ 70	−29	−13‡
	Cat pap m	+ 89	−39	−37
Epinephrine (low)	G P atrium	+210	0	0
Epi or Norepi (high)	G P atrium	+335	−19	0
Cardiac glycoside	G P atrium†	+605	0	0
(max inotropic level)	Cat pap m †	+500	0	0
Decreased frequency				
(from 60 to 6 per min)	G P atrium	− 60	0	0
Low Ca in medium	Cat pap m	− 86	0	0
Acetylcholine	G P atrium	− 94	0	0
Ryanodine	G P atrium	− 90	0	0
Cardiac glycoside	G P atrium	− 57	−64	−23
(toxic level)	Cat pap m	− 84	−66	−4

Zero denotes no statistically significant change
†After spontaneous failure In thes experiments levels in failure were used as control levels
‡Borderline statistical significance

lung preparations (especially those poisoned with phenylbutazone and fluoroacetate) also came to the conclusion that there was a good correlation between decrease in the work capacity of the heart (as determined by the competence index) and the decrease in content of CP rather than that of ATP The results of these investigators as well as those of others shown in table 2 suggest that CP may be more directly involved than ATP in supplying energy for contraction However it should be emphasized that all of these results were obtained on cardiac preparations in which oxidative metabolism was impaired experimentally and that other changes produced by impairment of metabolism—such as decreases in intracellular pH alteration in intracellular content of various ions and increases in intermediary metabolites—may have been more responsible for the decreases in contractile strength than the changes in CP (See also comments by Fawaz and Tutungi[19] on the lack of correspondence between cardiac output and CP levels in well oxygenated heart lung preparations poisoned with dinitrophenol)

Influence of Alterations in Experimental Conditions Which Do Not Inhibit Oxidative Metabolism

A large number of alterations in experimental conditions none of which is thought to act primarily by interfering with oxidative metabolism can produce marked increases or decreases in the contractile strength of cardiac muscle Among these are alterations brought about by additions of certain drugs by changes in the ionic content of the extracellular fluid and by changes in frequency of contraction In our laboratories we have investigated the levels of high energy phosphates of 2 isolated electrically driven cardiac preparations—namely the guinea pig left atrium and the cat papillary muscle—subjected to some of the alterations that influence contractile strength[3,4,10] Our findings are shown in table 3 In all cases in which similar alterations in experimental conditions were used on both preparations the results obtained were essentially the same

The upper part of the table shows the results obtained under experimental conditions that produced increases in contractile strength The only condition of this type

Table 4

Changes in High Energy Phosphates Associated with Experimental Failure

Type of preparation	Procedure of failure	% Change from control level CP	ATP	ADP	AMP	Reference
Guinea pig left atrium in vitro	Spontaneous	0	0	0	0	Furchgott and de Gubareff 19 8
Cat papillary muscle in vitro	Spontaneous	0	0	—	—	Lee et al 1960[4]
Dog HLP	Spontaneous	+ 6	0	—	—	Wollenberger 1947[20]
Guinea pig HLP	Extreme volume loading	− ⁰	−11†	+10†	—	Hochrein and Doring 1958
Rat heart in situ	Aortic constriction (acute failure 30 min)	−57	−16	—	—	Szekeres and Schein 1959[22]
Guinea pig heart in situ	Aortic constriction (chronic failure)	− 4	− 4	−40	0	Feinstein 1960
Dog heart in situ	Tricuspid valve avulsion plus pulmonary artery stenosis (chronic failure)	0	0	—	—	Olson and Piatneck 19 9[23]

Zero denotes no statistically significant change
†Average of only experiments Significance doubtful

under which there was an appreciable change in the level of high energy phosphates was that in which the K^+ concentration of the bathing medium was drastically reduced and then the change was a decrease rather than an increase All of the other experimental conditions (elevation of Ca^{++} concentration of the medium and addition of catecholamines or cardiac glycosides) led to marked increases in strength with small or insignificant changes in the levels of CP and ATP Our results with cardiac glycosides are in confirmation of Wollenberger s earlier results on the dog heart lung preparation [19]

The lower part of table 3 shows the results obtained under experimental conditions that produced decreases in contractile strength It is noteworthy that the reductions in strength brought about by varied procedures such as decrease of frequency of stimulation decrease of extracellular Ca^{++} addition of acetylcholine and addition of ryanodine were accompanied by no significant change of CP and ATP from control levels Previous to our work Wollenberger[20] and Fawaz and Hawa[11] had already found that local anesthetics and pentobarbital were able to impair the contractile strength of the dog heart lung preparation markedly without producing a significant fall in the high energy phosphate content of the ventricular muscle

The fall in contractile strength produced by cardiac glycosides at the toxic dose level was accompanied by significant decreases in both CP and ATP However this is not too surprising in view of the recent findings of Lee et al [1] that mitochondria obtained from hearts poisoned with a cardiac glycoside appear to have some degree of uncoupling of oxidative phosphorylation Thus the condition produced with toxic levels of cardiac glycosides may be an interference with oxidative metabolism which would be expected to lower the concentrations of high energy phosphates

The results shown in table 3 strikingly demonstrate that the strength of contraction of cardiac muscle may vary widely despite essentially constant levels of high energy phosphates The decreases in strength as one goes from those experimental conditions that produce marked positive inotropic effects to control conditions and from control conditions to those that produce marked negative

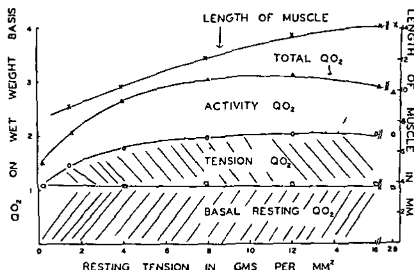


Figure 2

The effect of resting tension on the oxygen consumption of a papillary muscle stimulated at a frequency of 60 per minute. Basal resting QO_2 represents O_2 consumption of the unstimulated muscle at zero tension. Tension QO_2 represents the increment in consumption resulting from application of tension on unstimulated muscle. Activity QO_2 represents the increment in consumption owing to the contractile activity after stimulation at a given resting tension. (From Lee[25])

inotropic effects must therefore be attributed to deficiencies or impairments in the utilization of the available high energy phosphate stores for contraction.

Influence of Experimental Heart Failure

Three types of preparations have been used for concomitant studies of mechanical failure and high energy phosphate compounds in heart muscle. One type is the isolated cardiac preparation, such as the papillary muscle of the cat or the left atrium of the guinea pig, suspended in a physiologic medium and driven at a constant rate by electric stimulation. Such preparations will undergo spontaneous failure, as indicated by a loss of contractile force, in the course of several hours. A second type is the heart-lung preparation, which also will undergo failure spontaneously over a period of several hours even when there is no change in aortic resistance or venous reservoir pressure during the course of an experiment. This second type of preparation can also be made to go into failure at a faster rate by increasing the venous return (extreme volume loading) or at still a faster rate by increasing the aortic resistance. A third type of preparation is that in which failure is brought about in situ, either acutely or chronically, either by constricting the aorta or by constricting the pulmonary artery along with avulsion of the tricuspid valves. Chronic failure brought about by these procedures produces changes in the whole animal very similar to those seen in patients with congestive heart failure.

Table 4 gives the results of several investigations carried out with these different types of preparations. Acute spontaneous failure occurred in the isolated cardiac preparations[3, 4] and in the dog heart-lung preparation[18] with no decrease in high energy phosphate levels. On the other hand, acute failure in the guinea pig heart-lung preparation brought about by extreme volume loading[9] and acute failure of the rat heart in situ produced by marked constriction of the aorta[ ] were both associated with significant falls in high energy phosphates. In chronic heart failure initiated by stress on the right side of the heart, Olson and Piatnek[3] found no significant change in levels of high energy phosphates. However, in chronic heart failure initiated by stress on the left side of the heart, Feinstein did record significant decreases.[7]

In those cases of failure in which there was no significant change in high energy phosphate levels, there *is again a clear dissociation* of contractile strength and useful energy stores. Such cases of failure have therefore been attributed to a deficiency or impairment in the utilization of high energy phosphate stores for mechanical work.[3, 4] In the case of acute failure in the guinea pig

heart lung preparation with extreme volume loading and in the rat heart in situ with aortic constriction we feel that the fall in high energy phosphates may be largely due to the demand for oxygen to support an increased work load that exceeds the supply of oxygen delivered through the coronary circulation—thus leading to an adverse metabolic condition in the heart muscle

In chronic failure in guinea pigs studied by Feinstein in our laboratories a number of typical signs of marked congestive failure were apparent including cardiac hypertrophy pulmonary edema elevated venous and right ventricular pressures and elevated left diastolic pressures Thus it would appear that in chronic congestive failure associated with aortic stenosis there may be a considerable fall in high energy phosphate level Again the average fall of CP was much greater that that of ATP in these animals and there was a fair correlation between the severity of failure (estimated on the basis of physiologic and pathologic changes) and the extent of reduction of CP A second finding which should be mentioned and which was made by another graduate student in our department Arnold Schwartz was that the efficiency of oxidative phosphorylation (determined by P O ratios) of mitochondria from guinea pig hearts in congestive failure was depressed about 30 to 40 per cent below that of mitochondria from normal guinea pig hearts Thus there is a possibility that the low levels of high energy phosphates in guinea pig hearts in congestive failure may be due in part to a loss in efficiency of mitochondrial oxidative phosphorylation

The findings of Feinstein and Schwartz do indicate that an impairment of synthesis and a decrease in the steady state levels of high energy phosphate bonds may contribute to the severity of chronic congestive failure of guinea pigs with aortic constriction However it is impossible at present to state whether these changes played a primary role in producing the state of circulatory failure or whether they themselves developed only after the onset of physiologic and biochemical

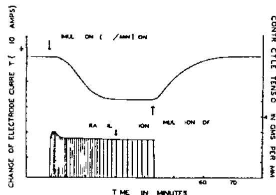


Figure 3

Changes in electrode current and contractile tension before during and after electrical stimulation of a papillary muscle Muscle diameter 0 32 mm wet weight 3 8 mg tension 2 Gm/mm² Electrode current measures O_2 tension in effluent from muscle The change in the current produced by stimulation can be used directly for calculating the rate of activity oxygen consumption (From Lee[25])

changes resulting from failure of the overloaded heart to maintain an adequate output Feinstein himself was able to show that the impaired function of the failing heart was not strictly dependent on the decreased CP content By injecting ouabain into guinea pigs in congestive failure he was able to improve cardiac function markedly and acutely (as judged from changes in intraventricular pulse pressures) even though the cardiac glycoside caused no increase in either CP or ATP above the low levels found in untreated animals

Changes in Efficiency in the Utilization of High Energy Phosphates for Contraction

In the 2 previous sections many examples of alterations in the contractile strength of cardiac muscle without significant alterations in high energy phosphate stores have been cited This type of finding has led to the conclusion that a decrease in contractile strength in one experimental condition as compared with another often results from a relative deficiency or impairment of the utilization of available high energy phosphates However the question is still left open of whether such a deficiency represents (1) a

decrease in the utilization of high energy phosphate bonds per contraction with little change in the efficiency of the conversion of the chemical energy in these bonds to mechanical energy; (2) a decrease in the efficiency of the conversion of chemical to mechanical energy with little change in the utilization of high energy phosphate bonds per contraction; or (3) a combination of both situations. To obtain a complete answer to this question one would have to determine simultaneously the strength of contraction and the amount of high energy phosphate bonds used in each contraction of the cardiac muscle under different experimental conditions. At present, however, there is no available method for determining directly the amount of high energy phosphate bonds used in each contraction and, therefore, an indirect approach must be employed.

The indirect approach developed by one of us (K. S. L.) involves the use of the isolated, electrically driven cat papillary muscle in an experimental set-up that permits the simultaneous measurement of contractile strength (usually isometric contractile tension) and rate of oxygen consumption (calculated from the fall in oxygen tension registered with a platinum oxygen electrode in a continuous-flow system).[3] With this set-up one can first obtain the resting oxygen consumption of the unstimulated quiescent muscle at a fixed resting tension and then the total oxygen consumption when the muscle is stimulated at the desired frequency at the same resting tension. The difference between the rates for total and resting oxygen consumption gives the rate of extra oxygen consumption required for contractions under the given experimental condition. From this rate and the frequency of contraction one can readily calculate the extra oxygen consumed per beat.

Bing[6] has stressed the need for using the activity oxygen consumption of cardiac muscle rather than the total oxygen consumption if one wishes to calculate the chemical energy used for the contraction process only. However, to obtain the activity oxygen consumption of the intact heart (or of the left ventricle) one must be able to determine its oxygen consumption in the arrested state with good coronary circulation and with intraventricular volume maintained at the mean diastolic volume of the active heart. These requirements are extremely difficult to meet, and the best procedure so far used for arresting the intact heart—namely, that of markedly increasing the K^+ concentration of the blood[7]—is open to some criticism.

In contrast to the experimental difficulties hindering the determination of activity oxygen consumption in the intact heart is the ease with which it can be determined in the isolated cat papillary muscle. Figure 2 shows both the resting and the activity oxygen consumption of cat papillary muscle, driven at a frequency of 60 per minute, as a function of resting tension.* Since the resting oxygen consumption, which is the sum of the consumption at zero tension ("basal resting oxygen consumption") and the extra consumption resulting from the application of tension ("tension oxygen consumption"), considerably exceeds the activity oxygen consumption at all tensions, it is apparent that the use of total oxygen consumption for the estimation of chemical energy required for mechanical work will give values that are much too high.

Figure 3 is a plot of data from a typical experiment in which the activity oxygen consumption and the contractile tension of a papillary muscle were determined simultaneously. The difference between the steady-state level of the oxygen tension curve during rest and during contraction at a fixed frequency can be used directly for calculations of the rate of activity oxygen consumption. By dividing the activity oxygen consumption per beat into the contractile tension at the steady-state level, one can obtain an index of the mechanical efficiency of the cardiac muscle under the experimental condition used.

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* Figures 2 and 3 reproduced from Lee: J. Physiol. 151: 156, 1960.[8] By permission of the Journal of Physiology.

Table 5

The Effect of Resting Tension on the Mechanical Efficiency of a Cat Papillary Muscle at Constant Frequency (60 per Minute)

Resting tension (Gm./mm.²)	Activity O_2 uptake per contraction (µL./mg. × 10⁻³)	Contractile tension (Gm./mm.²)	Index of mechanical efficiency
1	1.4	0.85	0.61
4	[illegible]	[illegible]	1.04
10	3.33	2	0.68
14	[illegible]	1.3	0.41

Index of mechanical efficiency was obtained in this and the next three tables by dividing value in third column by that in second column.

Table 6

The Effect of Frequency of Stimulation on the Mechanical Efficiency of a Cat Papillary Muscle at Constant Resting Tension (4 Gm. per Mm.²)

Frequency of stimulation per min.	Activity O_2 uptake per contraction (µL./mg. × 10⁻³)	Contractile tension (Gm./mm.²)	Index of mechanical efficiency
10	3.20	0.4	0.125
30	2.65	1.3	0.49
90	1.76	2.1	1.19
130	1.18	0.9	0.76

It is postulated in this discussion that the activity oxygen consumption is a measure of the extra oxidative energy required for the synthesis of those high energy phosphate bonds used for contraction and that the index of mechanical efficiency obtained as outlined above is a measure of the efficiency for conversion of the chemical energy available in these bonds into mechanical energy in the form of work. It is also postulated that a comparison of indices of efficiency under different experimental conditions will enable one to determine whether the efficiency of this chemical-mechanical conversion is altered. These postulates are valid only if the 4 following assumptions are valid: (1) that the efficiency of oxidative phosphorylation remains constant; (2) that the resting oxygen consumption which meets the needs of all energy requiring processes other than contraction is the same for the quiescent and for the stimulated muscle; (3) that oxidative metabolism is essentially the exclusive means of energy production and that glycolysis is insignificant; (4) that the force of isometric contractions is practically proportional to work which would be done in isotonic contraction. It must be admitted that at present there is no experimental evidence available to either prove or disprove the validity of assumptions (1) and (2). On the other hand the validity of assumption (3) is supported by preliminary work with papillary muscles as well as by older experiments showing that lactic acid is not produced in beating hearts under good aerobic conditions.[6] Finally, recent work in this laboratory using papillary muscles under isotonic rather than isometric conditions supports assumption (4) that isometric contractile force is proportional to isotonic work as long as the resting tension on the muscle is the same under both conditions.

A number of examples of comparisons of indices of efficiency under different experimental conditions that lead to marked alterations in force of contraction are shown in tables 5, 6, 7 and 8. These examples, all of which represent experiments on single papillary muscles, come partly from published work of K. S. Lee and partly from unpublished work. In all of the examples given there are marked decreases in efficiency under those experimental conditions associated with decreases in contractile tension.

Tables 5 and 6 show the effects of variations in resting tension at constant frequency of stimulation (60 per minute) and the effects of variations in frequency at constant resting tension. In the experiment at constant frequency, efficiency reaches a maximum at a resting tension of 4 Gm./mm.² and then falls off at higher tensions. The finding of an intermediate resting tension at which the index of mechanical efficiency is highest is not too surprising in view of earlier work on the intact heart which indicated that the ratio of measurable external work to total oxygen consumption is maximal at an intermediate level of left ventricular diastolic volume.[8]

In the experiment at constant resting tension (table 6), the index of mechanical effi

Table 7

The Change of Mechanical Efficiency of a Cat Papillary Muscle During Failure In Vitro and after Recovery with Ouabain

Experimental condition	Relative activity Q_{O_2}	Relative contraction	Relative index of mechanical efficiency
Prior to onset of failure	1	1	1
During failure	0 [illegible]0	0 3[illegible]	0 3
Recovery from failure with ouabain (initial therapeutic stage)	0 91	0 [illegible]1	0 [illegible]8

Relative rather than absolute values are used in this and the following table

Table 8

The Effect of Ca^{++} Concentration in the Medium on Mechanical Efficiency of a Cat Papillary Muscle

Ca concentration of medium	Relative activity Q	Relative contractile tension	Relative index of mechanical efficiency
No Ca (early stage)	0	0 06	0 [illegible]7
Ca 1 2 × 10 M	0 [illegible]1	0	0 [illegible]0
Ca 4 × 10 M	1	1	1
Ca [illegible] × 10 M	1 [illegible]1	1 4	1 16

ciency rises steadily with increasing frequency up to about 90 beats per minute and then falls off as the frequency is further increased The papillary muscle like a number of other cardiac preparations including isolated guinea pig and rabbit atria exhibits an increase in contractile tension with increasing frequency over a fairly wide range of frequencies This well known positive staircase phenomenon is apparently the result of an increase in mechanical efficiency with increase in frequency and not the result of an increase in utilization of chemical energy Indeed on the basis of the activity oxygen consumption per contraction it appears that the chemical energy utilization per contraction actually decreases as the frequency increases If the postulate that the index of mechanical efficiency is a direct measure of the efficiency of conversion of phosphate bond energy into mechanical energy is correct then the marked decrease in contractile strength at low frequencies may be attributed to a very low efficiency for this crucial conversion

In a previous report one of us (R F F) suggested that the positive staircase effect in isolated guinea pig atria was dependent on the rate of some activation process occurring between beats and that the degree of activation of the cardiac muscle at the time of an action potential determined the size of the contractile response initiated by the action potential [9] It was proposed that at low frequencies associated with small contractions the rate of the activation process was so slow that the degree of activation attained by the time of the next action potential was relatively small and that at high frequencies associated with large contractile responses the rate of the activation process was very much faster so that the degree of activation at the time of the next action potential was relatively great In view of the present findings it appears that this proposed activation process may in reality be a process of restoration of a state in the muscle that determines the efficiency of the conversion of chemical to mechanical energy

In table 7 are the results of an experiment on spontaneous failure and recovery from failure after addition of a cardiac glycoside It is apparent that the failure is due primarily to a loss in mechanical efficiency rather than to a loss in energy production and that the recovery from failure with ouabain is due to an increase in mechanical efficiency rather than to an increase in energy production These results also are not too surprising in view of older work on *intact hearts which indicated that certain* types of failure were associated with a decrease in the ratio of measurable external work done by the heart to total oxygen consumption of the beating heart and that recovery from failure with cardiac glycosides was associated with an increase in this ratio [30] However it was impossible to separate activity oxygen consumption from total oxygen

consumption in these earlier studies and thus the changes in overall efficiency could not be claimed to demonstrate directly changes in the efficiency of the conversion of chemical to mechanical energy in the contraction process alone. The present findings on the other hand do support strongly the concept that there is a loss of efficiency in this conversion in spontaneous failure and a recovery of efficiency during the restorative action of cardiac glycosides.

Table 8 shows the results of an experiment in which the calcium content of the incubation medium was varied over a wide range. With increases in calcium there is an increase in activity, Q_0, contractile tension and the index of mechanical efficiency. From the data one may conclude that the increase in contractile tension with increase in calcium is due in part to an increase in chemical energy utilization per contraction and in part to an increase in efficiency of the conversion of this energy into mechanical energy. Thus the reduction of the contractile force brought about by reduction of the extracellular calcium is probably attributable both to a decrease in the utilization of high energy phosphate bonds per contraction and to a decrease in efficiency in the conversion of the chemical energy of those bonds used into mechanical energy.

From the results of experiments such as those discussed in this section we may conclude that in many experimental conditions in which a decrease in contractile force has been attributed to a deficiency in the utilization of high energy phosphates for contraction, the deficiency is in large part, and sometimes almost exclusively, due to a loss in efficiency in the conversion of chemical energy of high energy phosphate bonds into mechanical energy. This conclusion is admittedly based on interpretations the correctness of which depends on the validity of certain assumptions that have not yet been strictly proved. However the conclusion presents an interesting working hypothesis and confronts us directly with the problem of what factors control the efficiency of utilization of high energy phosphates for mechanical work in heart muscle. Speculation about these factors is beyond the scope of the present paper.

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Discussion

Dr R E Davies (Philadelphia Pa) It seems that we and Dr Mommaerts have been floundering in the same morass for 8 years or so Concerning the question of whether the creatine phosphate ATP system changes during a single twitch he has announced that it does that it doesn t and now again that it does Originally we found that it did not Then about 3 years ago we found a very labile compound which we called XP that was present in resting muscle and not in contracted muscle After announcing this discovery we kept on working and found that sometimes it could be seen and sometimes it couldn t Finally we decided that XP was a will o the wisp—or a Wilfried o the wisp

We that is Drs Cain Delluva Kushmerick and I then stopped trying to find an unknown compound and tested known compounds to find the source of the inorganic phosphate we had found to be liberated in a single twitch The content of each compound we tried—however esoteric and unstable—remained unchanged Finally despite Wilfried s published results we determined the creatine content and found that it did change Then we learned from Wilfried that recently he too had had inconsistent results In seeking the cause for this inconsistency we examined the dates when the experiments had been done We found that every time the creatine content hadn t changed the experiments had been done in spring or summer and every time it had changed the experiments had been done in the fall or winter Whenever XP was there and changed it was spring whenever it either wasn t there or didn t change it was fall Perhaps this seasonal variation means that there may be an exceedingly labile compound that can be detected only in the spring or summer at other seasons it may change and be reconstituted so quickly that we miss it

Thus we must wait for spring or go to South America or start injecting frogs with pituitrin or perhaps choose an animal that doesn t have this seasonal variation Then maybe in another 2 years we will find that we are just where we are today

Dr Kuang Soo Lee I should like to ask Dr Mommaerts a question You mentioned that there is a possibility that heat of activation and heat of shortening and heat of work might come from different metabolic pathways Is there any experimental evidence that even suggests this?

Dr Mommaerts Not so far As I implied in most of the experiments the only detectable breakdown of phosphocreatine liberates equivalent amounts of phosphate and creatine There are some circumstances in which a sizable degree of phosphorylation takes place as well with hexose phosphate formation—less phosphate than creatine is liberated For a season in which the formation of hexose phosphate was relatively prominent we plotted either the fraction that goes to phosphate or to hexose phosphate as a function of the total shortening there was no correlation whatsoever So far then we have had no indication of different chemical reactions serving 1 purpose or the other

Dr Podolsky There are some early experiments of A V Hill which suggest that there is only 1 driving chemical reaction (*Proc Roy Soc London s B* 127 297 1939) He defined *initial energy* as the sum of the work done and the heat liberated while the muscle is active The *recovery heat* is the heat liberated while the muscle recovers from activity The initial energy is liberated quickly compared with the recovery heat so these quantities are experimentally separable The *total energy* is initial energy plus recovery heat that is the total energy exchange associated with activity

Hill measured energy exchanges under a variety of conditions of mechanical loading and under all these conditions the ratio of intial energy to total energy turned out to be the same This should be the case if the driving chemical reaction for the contractile mech

anism were the same under all conditions of loading. Is this clear?

Dr Lee Yes but I don't think it really can be considered as evidence because thermodynamically whatever it produces no matter what pathway you take it is compensated for eventually

Dr Podolsky I think it does constitute evidence for the following reason If there were 2 different reactions each having different heats of reaction and if under certain conditions 1 of them proceeded to a greater extent than the other then Hill would not have found regularity in the ratio of initial energy to total energy

Dr Huxley May I ask Dr Podolsky if he can make any estimates of the number of substrate molecules that need to be bound to the actin filament to produce the necessary tension?

Dr Podolsky I am afraid that I can not provide you with an answer

Dr Huxley I was wondering if it would have to be the same sort of number as Morales (Morales and Botts *Arch Biochem* 37 283 1952) had to employ in his mechanism in which it was ATP binding that gave rise to the change on the electrostatic model Actually in that model you need a tremendous amount of ATP and if in your model you wipe that amount of ATP off the actin each time it would imply you have to split that much every time

Dr Podolsky The difficulty with a calculation for the melting model is that one needs a good theoretical basis for relating the extent of binding to force Although this can be done in principle I have not yet found out how to do it

Dr Palade You considered 2 possibilities in contraction Dr Podolsky—the sliding and the folding of the actin filaments How extensive should this folding be to account for the contraction and is the folding of such a magnitude that it should be easily visible in the electron microscope? In other words is the folding mechanism still compatible with the present morphologic evidence?

Dr Podolsky The extent of folding of course should be the same as the extent of shortening Now, the way to do the electron microscope experiments—and some people have tried this—is to quick freeze the muscle while it is shortening physiologically and then make sections In both models the filaments should return to their original length when the muscle relaxes The question is whether you can catch the folding before the thin filament has had a chance to relax again Do you see what I mean?

Dr Palade Yes but there are micrographs indicating for example that the contraction has gone to the extent that the I bands have disappeared and the filaments are still more or less of the expected length on the basis of sliding

Dr Podolsky I think that should happen at the end of contraction in both cases The question is what generates the shortening while the muscle is contracting not what is the disposition of the filaments after the contraction has ended

Dr Palade So by the time the preparation is connected the filaments are back to their original length?

Dr Podolsky Yes that is a way of getting around it

Dr John Gergely (Boston Mass) Dr Furchgott in connection with this puzzling problem of failure in efficiency is there any possibility that the lower tensions one obtains under various conditions are due to the block in the excitation coupling i e is there an inefficiency in transmitting the stimulus to the contractile system? Also in the cases in which efficiency in terms of oxygen consumption is decreased as compared with the development of tension is one dealing with an uncoupling at the level of generation of phosphatase?

Dr Furchgott First about excitation coupling It is quite possible that a block or impairment of this little understood primary process accounts for the decreased contractile force under some experimental conditions This may be the situation in the case of the negative inotropic action of acetylcholine on atria since the markedly shortened action po-

tential may not suffice to give full excitation of the atrial cells Also the reduction in force at low extracellular Ca^{++} concentrations may be due in part to an impaired excitation coupling However I would not expect an impairment of this primary process to cause a marked decrease in mechanical efficiency (as indicated by a marked fall in the ratio of mechanical work to activity oxygen consumption) such as we found on reducing frequency or allowing spontaneous failure to occur in papillary muscles

As for the question of whether an uncoupling at the level of generation of high energy phosphate may account for the decreased efficiency I think the answer is that such uncoupling of oxidative phosphorylation can probably be ruled out in certain cases of decreased efficiency that we have studied—namely those involving low tension low extracellular Ca^{++} and spontaneous failure In all of these cases the resting oxygen consumption was about the same or somewhat lower than that under control conditions whereas a greater resting oxygen consumption would have been expected if there was uncoupling at the level of generation of high energy phosphates

Chairman Taggart I think Dr Mommaerts has been waiting to speculate on this

Dr Mommaerts Yes I feel very definitely that it has to do with excitation contraction coupling but probably in a way that is not revealed by the duration of the action potential Dr A Y Brady (unpublished data) has felt that in the heart (not in skeletal muscle) there is a definite correlation between the duration of the action potential and the duration of the active state It is as if the action potential or its plateau turns the active state on or off

However all the inotropic changes that we have encountered in the heart are not primarily caused by a change in the duration of the active state They are caused by a change in the force velocity relation so that within the same available time a greater or smaller fraction of the active state is realized in terms of external tension I don t know whether any patients are going to be helped directly by this knowledge but one can say that heart failure is a disease of the constant b or perhaps of the constant a in the Hill equation

Dr Hoffman Dr Mommaerts I wasn t sure if the variation in the constant for the activation was primarily a function of resting length resting tension or contractile tension If the variability is primarily a function of resting length is it related in any way to the sarcomere length or let s say to the extent of overlap of thin and thick filaments in a given sarcomere?

Dr Mommaerts It may very well vary Dr Hoffman with the resting length but I also have in mind the variation with the contracting length Among others Aubert has studied extensively the maintenance heat as a function of the contracted length (Le couplage energetique de la contraction musculaire Bruxelles Editions Arscia 1956) Over certain ranges there is a linear relation between maintenance heat and tension One enters here into a form of philosophy which considers that getting a linear relation is the highest purpose of the scientist Accordingly one might believe that it is the tension during activity that has a primary influence upon the maintenance heat

Of course tension and length are both changed and apart from the linearity who is to tell which of these factors is of primary importance in determining the maintenance metabolism or even whether this is a valid question? There is however a very real operational difference between the 2 concepts which appears when evaluating the energy mobilized in a tetanus of constant duration in which a given degree of shortening is allowed In one instance with a light load the shortened length is reached rapidly in the other with a heavy load slowly Which value of the maintenance heat will be operative? That determined by the varying length or that determined by the tension? The answer is not available but the distinction may well be accessible to experimental analysis

Dr Podolsky I have had a chance to think about the earlier questions a bit more In re

ply to Dr Huxley, one reason Morales (Morales and Botts *Arch Biochem* 37 283 1952) needed so many particles to bind to the polymer is that in an electrostatic mechanism the force is reduced by the ionic strength of the milieu and in muscle the ionic strength is quite high On the other hand the polymer melting process need not be a function of ionic strength (although it may) so there is a chance that it might not require nearly so many particles to generate the force This could be especially true if the polymer had a high degree of crystallinity in this case melting is a cooperative process

About Dr Palade s question although there is no published electron microscope evidence that supports folding there is some physiologic evidence In both the sartorius muscle (experiments of Buchthal and Kaiser *Dan Biol Medd* 21 121 1951 and of Marechal *Arch internat physiol* 63 128 1955) and in the heart (experiments of Rosenbleuth and Rubio *Arch internat physiol* 68 181 1960) it was found that if shortening starts from beyond a certain length the muscle has a memory The force developed at a shorter length depends on whether that length was reached by active or passive shortening This phenomenon is known as hysteresis in the length tension curve In the sliding model, the shortening muscle should have no memory but in the folding model it could have a memory because there is a point of reference —the point of attachment of the thin filaments upon activation

In the case of the frog sartorius muscle there is no memory if the initial length is less than that length at which the H zone vanishes that is if the thin filaments meet at the center of the A band However at these lengths you would not expect it to have a memory because now the ends of the thin filaments would always attach at the same point the center of the A band Although the argument is indirect this physiologic evidence can be taken to support a folding rather than a sliding model

Chairman Taggart I am sure that this discussion could go on through the night It is quite evident I think to all of us in the audience that the mechanochemical features of muscle contraction are not yet thoroughly elucidated I should like to thank our speakers for their very illuminating discussions and our audience for their attention and participation

The Rewards of Scientific Investigation

It is stranger that we are not able to inculcate into the minds of many men the necessity of that *distinction* of my Lord Bacon s that there ought to be experiments of *light* as well as of *fruit* It is their usual word *What solid good will come from thence?* They are indeed to be recommended for being so severe *exactors* of *goodness* And it were to be wished that they would not only exercise this vigour about *experiments* but on their *lives* and *actions* that they would still question with them elves in all that they do what *solid good* will come from thence? But they are to know that in so large and so various an *art* as this of *experiments* there are many degrees of usefulness some may serve for real and plain *benefit* without much *delight* some for *teaching* without apparent *profit* some for *light* now and for *use* hereafter some only for *ornament* and *curiosity* If they will persist in contemning all *experiments* except those which bring with them immediate *gain* and a present *harvest* they may as well cavil at the providence of God that he has not made all the seasons of the year to be times of *mowing reaping* and *vintage*—T Sprat *The History of The Royal Society of London* Ed 3 1[illegible] Cited by W M Bayliss in the preface to *Principles of General Physiology* Ed 4 London Longmans Green and Co 1924 p xvi

III Hibernation in Animals

Chairman Alfred P Fishman, M D

Introduction

By Alfred P Fishman M D



Figure 1

TO THE interested bystander the subject of hibernation is a curious mixture of mystery and of science Part of the mystery stems from the haze of uncertainty that surrounds the idea of suspended animation part from the exotic creatures that indulge in hibernation As a prelude to Dr Lyman s scientific discussion of hibernation in mammals I should like to remind you of some of the creatures with which he will probably deal

Every group of vertebrates except birds hibernates But of all the hibernating mammals the hedgehog and the dormouse have emerged as the most popular subjects for study in the laboratory

The hedgehog is the less familiar of the two It is a mammal of the order Insectivora ordinarily about 10 inches long In figure 1 is illustrated a typical hedgehog on the verge of hibernation It may be seen that its appearance is characterized by a surface of spines and a short tail Not evident is its poorly developed brain When startled or threatened it rolls up into a ball from which spines protrude in all directions

From the Department of Medicine College of Physicians and Surgeons Columbia University New York New York

In contrast to the hedgehog the dormouse is a friend of long standing It is shown in familiar surroundings in figure 2 Not manifest in this illustration is the fact that it is a small arboreal squirrel like rodent which is more apt to be found in bushes and trees than at tea parties But by the close of the summer festivities when it has gorged itself to extreme obesity it curls up into a ball and hibernates until Spring As will be recalled from figure 2 the dormouse may be roused from its torporous state only to lapse back into suspended animation when external stimulation ceases

It is to subjects such as these that Dr Lyman has devoted much of his scientific life Dr Lyman has won world renown for his fresh observations and ingenious experiments By training he is a comparative anatomist who has exploited the special technics of physiology and biochemistry to unravel some of the mysteries of hibernation in mammals He will review for us in the light of his own researches the present understanding of the biology of hibernation



Figure 2

Hibernation in Mammals

By Charles P. Lyman, A.B., M.A., Ph.D.

Hibernation and enforced hypothermia in mammals are widely different physiologic states. Prior to hibernation there are various preparations for the hibernating state including polyglandular endocrine involution, fattening and/or food storage, and changes in the saturation of depot fat in some animals. The actual causes for the onset of hibernation are unknown, for most hibernators can remain active at low environmental temperatures for long periods. Entrance into hibernation is under precise physiologic control, with heart rate, respiratory rate, and oxygen consumption slowing before a decline in body temperature. A reasonably high blood pressure is maintained during this period and in deep hibernation by an increased peripheral resistance produced in part by vasoconstriction. Homeostasis is continued in hibernation as evidenced by a normal blood pH, a sensitivity to inspired CO₂ and a response to ambient temperature below 0 C by increased metabolic rate. At any time during entrance into hibernation or during hibernation the animal may arouse from this condition. Arousal is a coordinated physiologic event in which the anterior of the body is warmed rapidly by shivering and other heat generating mechanisms, while warmed blood is shunted from the posterior by differential vasoconstriction until the anterior reaches nearly 37 C. The tissues and organs of mammals that hibernate are capable of useful function at lower temperatures than the tissues of mammals that do not hibernate, but a hypothermed mammal that can hibernate will die in hypothermia even though it lives longer and at a lower temperature than a mammal that can not hibernate. Hibernation must involve a resetting of the "physiologic thermostat," which thus permits a controlled cooling of the animal, but the nature of this "resetting" is not known.

THE BURGEONING INTEREST in hypothermia for surgery has aroused some curiosity among physicians in the natural hypothermia that occurs seasonally in mammals that hibernate. There can be no doubt that natural hibernation as practiced by many bats, rodents, and insectivores is a far less traumatic experience than the hypothermia that is forced on experimental animals, and a study of the former may help to clarify difficulties encountered in the latter. Quite obviously, both hibernation and hypothermia have in common a profound lowering of the usual body temperature of about 37 C, but beyond this similarity it is apparent that hibernation in all its phases is a controlled and remarkably regulated condition, while hypothermia consists virtually in a breakdown of temperature regulation that causes a weakening or collapse of other homeostatic mechanisms.

It has often been stated that mammals which hibernate have an inadequate system of temperature regulation so that when exposed to cold their temperatures decline and the animals enter the hibernating state. Actually, however, most hibernators that are not prepared for hibernation can stay active and healthy for months or even years at temperatures that are often fatal to the common laboratory animals of the same size.[1] If, on the other hand, the potential hibernator is prepared for hibernation at the time it is exposed to cold, it may enter the state of hibernation within 24 hours.

Preparation for Hibernation

The nature of this preparation for hibernation is not clearly understood, but it is certain that the animal must be ready for hibernation or it will not hibernate. Most of the ground squirrel family become extremely

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fat as the season for hibernation approaches but this does not necessarily seem to be correlated with an abundance of succulent foods There is some evidence that the fatter animals hibernate before the thin ones but fat or thin they all eventually hibernate during the fall months and come out of the hibernating phase of their yearly cycle when spring arrives The length of daily illumination has little if any effect upon this cycle and the evidence to date indicates that the cycle is innate and receives few if any clues from the environment

Unlike the ground squirrels the golden hamster is not truly cyclic and if exposed to cold for sufficient time will enter hibernation at any time of year Hamsters store large quantities of food prior to hibernation If denied the ability to store hibernation is delayed as if there were some safety check that will not permit the animal to hibernate without adequate supplies for the winter [3] Hamsters lose fat when exposed to cold prior to hibernation and the fat that remains is less saturated and hence has a lower melting point than the fat from animals kept in a warm environment (fig 1*) [4] This would seem like a nice mechanism to maintain fat in a liquid condition during hibernation but we have been able to show in both hamsters and ground squirrels that hibernation is not delayed when animals are fed a diet that results in depot fat so saturated that it is actually solid in the hibernating animal Contrariwise the onset of hibernation is not accelerated if the animals are fed a diet that results in body fat with a low melting point

In the small rodent hibernators lack of nourishment may rapidly induce hibernation The metabolic budget of a diminutive mammal is extreme for the high surface to mass ratio results in a disproportionately large heat loss When denied metabolic fuel the North American pocket mouse *(Perognathus)* enters the hibernating state and thus reduces its immediate metabolic problem This is indeed a hibernation of desperation for the food supply may be as bad when they awaken as when they entered hibernation Other small hibernators notably bats and the European birch mouse *(Sicista)* [6] usually allow their body temperature to drop any time they become inactive though on other occasions they remain warm

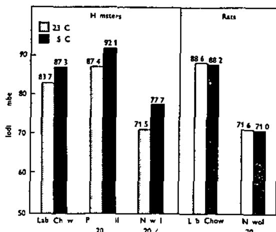


Figure 1

Effect of old exposure on the saturation of depot fat of hamsters and rats on various diets Bar graph on right of each pair is the iodine number of the fat of animals exposed to cold Neuol is a highly saturated edible fraction of beef tallow (From Faucett and Lyman [4])

Apart from the condition of the animal as far as available food is concerned there is evidence that the endocrine glands play a role in the preparation for hibernation Histologic studies have shown that prior to hibernation all the endocrine glands show a marked decrease in activity Hence it has been postulated that a polyglandular involution must take place before the animal can hibernate [7] The precise importance of the various endocrines in setting the stage for hibernation has not been elucidated and some of the observed endocrine involution may be simply incidental For example the gonads of most hibernators involute right after the breeding season which is several months before hibernation occurs Moreover it seems reasonably certain that no single endocrine

F gure 1 reproduc l from Fawcett and L n an J Phv iol 1 6 35 1954 Bv permission of the Journal of Phys ology

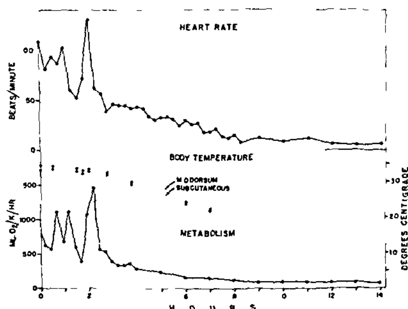


Figure 2

Record of woodchuck entering hibernation showing chilling and rewarming during the first 2 hours then a steady decline into hibernation Heart rate drops first followed by a decrease in oxygen consumption and then in body temperature Heart rate and oxygen consumption increase before body temperature when animal rewarms (From Lyman[25])

gland controls the hibernating state for removal of any one of them does not hasten the onset of hibernation

Hibernation

In spite of its importance in the study of hibernation the process of entering the hibernating state has been poorly understood This is because the onset of hibernation is capricious and recording of the various changes involves instruments that can monitor a chronic preparation over long periods Such instruments were unavailable a decade ago It is natural to assume that a decline in body temperature would be the first indication of hibernation and that other vital functions would diminish in rate according to the van t Hoff rule as the temperature dropped Such is not the case for respiratory rate *heart rate and oxygen consumption are all* reduced before a detectable drop in body temperature occurs (fig 2*) The contrast between this and enforced hypothermia is striking In the hibernating animal, hibernation occurs passively as if, as Prosser has so aptly stated someone had ‘turned down the thermostat ’[18] In enforced hypothermia the animal chills in spite of a violent metabolic effort to remain warm

The actual entrance into hibernation is under fairly rigid physiologic control, and the decline in body temperature is always slower than it would be if the thermostat were quickly changed from a setting of 37 to 5 C In most of the hibernators bouts of *shivering often* accompanied by gross muscular movements take place from time to time On these occasions the heart speeds and the metabolic rate increases If the bouts are of long duration the body temperature ceases to drop and often rises transiently The result is that the animal may enter hibernation by uneven steps rather than in a smooth curve At least one hibernator the California ground squirrel, allows its body temperature to drop only part way toward the deeply hibernating state on its first attempt With each subsequent entrance the body temperature drops to a lower level until it finally reaches a body temperature slightly above the ambient temperature of 5 C (*fig 3†*) It has been *suggested that* these precisely regulated drops serve to test the state of the animal so that it never

*Figure 2 reproduced from Lyman Am J Physiol 194 83 1958[25] By permission of the American Journal of Physiology

†Figure 3 reproduced from Strumwasser Bull Mus Comp Zool 124 85 1960 By permission of the Museum of Comparative Zoology

lowers its body temperature below a level for which it is physiologically prepared[9] Furthermore each entrance into and arousal from hibernation in this animal takes place at a precise time of day even in the absence of external clues which suggests that the onset of hibernation is being controlled by some sort of an internal clock

The circulation during entrance into hibernation in the thirteen lined ground squirrel is regulated with considerable precision The first precipitous decline in heart rate signaling the start of the whole process causes a drop in blood pressure which however remains within the normal values for active animals (fig 4*) The heart continues to slow both by skipping beats and by reduction in the number of even beats (fig 5) As hibernation deepens this becomes more and more exaggerated until in deep hibernation the heart rate can be as low as 3 beats a minute with periods of bradycardia lasting 30 seconds or more (fig 6) Peripheral resistance as indicated by the slope of the diastolic run off increases with the decline in body temperature so that the mean blood pressure remains at remarkably high levels for such a slow heart rate Undoubtedly part of the increase in peripheral resistance is due to the increased blood viscosity at low temperatures but some of it must also be caused by vasoconstriction for vasodilators and adrenergic blocking agents cause a decrease in peripheral resistance accompanied by an increase in heart rate that may be in part compensatory (fig 7) Since the temperature in every part of the body declines at an equal rate (see figs 2 and 4) it is apparent that the vasoconstriction is generalized and not confined to certain organs or areas[10]

The preferential ambient temperature for hibernation in most mammals is a few degrees above the freezing point of water As the body temperature approaches that of the environment the body temperature curve be-

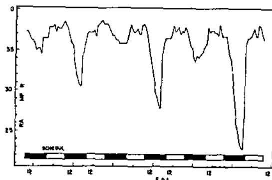


Figure 3

Brain temperature of a California ground squirrel entering hibernation in the cold during the summer showing 3 test drops M and N=midnight and noon (From Strumwasser[9])

comes asymptotic and if the ambient temperature does not suddenly rise the body temperature always remains slightly higher than the environment The hibernating mammal is curled in a tight ball, with only the back exposed in the nest and the low metabolic rate is enough to maintain this temperature difference Bats which must roost and hibernate in an extended position have a body temperature identical with the environment if they are forced to hibernate singly

In deep hibernation the metabolic rate is often less than one fiftieth of that of the awake animal at rest and the body temperature may be as low as 3 C In spite of this the animal maintains a remarkable degree of homeostasis Although the blood sugar is low in some species during hibernation it is normal in others The pH is essentially normal and pCO_2 is low compared to the active animal In hibernation the respiratory centers remain remarkably sensitive for an increase of ambient CO_2 above 4 per cent will cause an increase in the respiratory rate The hibernating animal also retains a certain degree of homeothermism Between ambient temperatures of about 4 and 15 C the body temperature passively follows the temperature of the environment If the ambient temperature slowly drops to 0 C or lower the metabolic rate is increased and body temperature is maintained above the freezing point Some

Figures 4 5 6 7 9 reproduced from Lyman and O Brien Bull Mus Comp Zool 1 4 353 1960[10] By permission of the Museum of Comparative Zoology

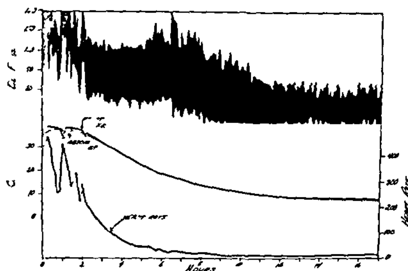


Figure 4

Blood pressure (mm Hg) heart and abdominal temperature and heart rate of a thirteen lined ground squirrel entering hibernation Blood pressure in dark area is highest systole and lowest diastole recorded for a 1 minute period at 5 minute intervals Heart rate and blood pressure decline before body temperature Heart remains slightly warmer than abdomen in hibernation (From Lyman and O'Brien [10])

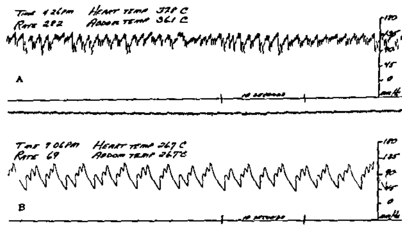


Figure 5

A Blood pressure and EKG of animal graphed in figure 4 Uneven pattern of beats is typical for this stage 4 26 p m =1½ hours on figure 4 B Same animal later Pattern of beats and skipped beats is now even (From Lyman and O'Brien [10])

times the animal remains in hibernation with a higher metabolic rate and on other occasions the metabolic effort is sufficient to cause arousal from hibernation In other cases this rather low grade temperature regulation is insufficient to protect the animal and it freezes to death in hibernation [11]

Deep hibernation does not last throughout the winter in any mammal for periodically the animal wakes eats stored food if available voids and returns to the hibernating state The periods of arousal vary with the species Hamsters generally hibernate for only 3 to 5 consecutive days ground squirrels for a few days longer and bats may hibernate for a month or more without waking

During the periods of hibernation cell growth and replacement are greatly reduced but not completely stopped Although it has been reported that mitotic activity is in abeyance during hibernation [1] we find mitotic figures in the crypts of Lieberkuhn of ground squirrels that have been hibernating continuously for as long as 13 days Radioactive iron injected into continuously hibernating hamsters is found in the erythrocytes showing that hematopoiesis is continuing albeit at a very slow rate The reduced rate of cell production seems to be paralleled by a slow rate of cell aging and destruction for erythrocytes tagged with chromium remain in the circulation for a much longer time in a hibernating animal than they do in an animal that is active [13] No experiment has been designed which proves that an animal in hibernation lives longer than its active litter mate

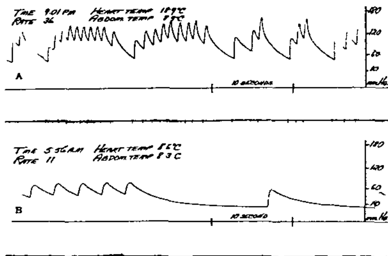


Figure 6

A Same animal as figures 1 and 5 Transient increase of heart rate at low body temperature Note muscle action potential on EKG denoting shivering
B Same animal now in deep hibernation Blood pressure slightly damped Blurring of EKG is electrical artifact (From Lyman and O Brien [10])

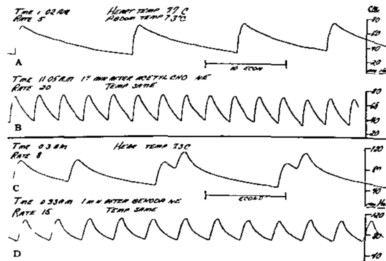


Figure 7

A Pulse pressure in deep hibernation
B Effect of acetylcholine Faster diastolic run off from slightly lower systolic pressure than in Figure 7A
C Pulse pressure in deep hibernation
D Pulse pressure after adrenergic blocking agent Faster diastolic run off from same systolic pressure (From Lyman and O Brien [10])

but it is interesting that bats which may spend one half of their life in hibernation are extremely long lived animals for their size

Earlier clinical work had suggested that neoplastic tissue might be differentially killed or its growth slowed by cold and hibernating animals provided an excellent opportunity to test this premise Homologous tumors implanted in the cheek pouch of hamsters which then enter hibernation showed no detectable increase in size during the period of hibernation though on microscopic examination some of the cells appeared to be viable As soon as the animals awoke from hibernation however growth of the tumors resumed [15] Heterologous human tumors implanted in the same manner also were not destroyed by the 5 C temperature of hibernation and lived to grow again when the animals awoke [1]

Animals exposed to radiation during hibernation show little or no cellular damage as long as they remain in the hibernating state Once they have aroused from hibernation the cell destruction begins and the length of the animal s life is only prolonged by the number of days it has been in hibernation [16] The nature of this radiation memory is not understood and its study may be a help in the clarification of the processes involved in radiation injury Reparative processes such

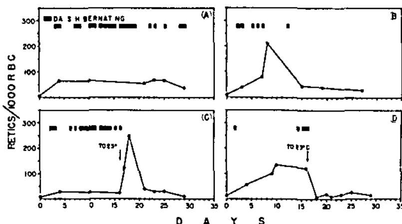


Figure 8

Effect of hibernation on the reticulocyte response of individual hamsters at 5 C Animals bled on day zero Hibernation delays and suppresses the response (From Lyman et al [17])

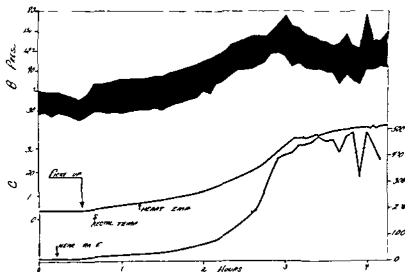


Figure 9

Thirteen lined ground squirrel waking from hibernation graphed as in Figure 4 Note difference in heart and rectal temperature Blood pressure drops as rectal temperature rises (From Lyman and O Brien [10])

as hematopoiesis after bleeding take place at an extemely slow rate if at all during hibernation and it is only after waking that the animal reacts to the stimulus for repair (fig 8*) [17]

Arousal

Continuous hibernation throughout the winter months would be the most efficient way to avoid an unfavorable situation but this does not occur in any species that has been studied Although many theories have been advanced the cause for periodic arousals is not understood Arousal may be evoked from an animal in hibernation at any time if a sufficiently strong stimulus is applied but the strength of the stimulus necessary to produce the arousal seems to vary with the species and the physiologic state of the animal at the time The process of arousal is a coordinated series of physiologic events in which the hibernator returns to the active state in a minimum of time using only heat generated by its own body In this orderly sequence the control of the circulation is remarkably exact and it is this circulatory control that permits the rewarming with such efficiency Arousal from hibernation is in sharp contrast to the condition of an animal in enforced hypothermia where the ability to rewarm is almost completely lost

The process of arousal is apparently the same whether the awakening is evoked by an external stimulus or whether it occurs naturally during the hibernating cycle One of the first signs of awakening is an increase

Figure 8 reproduced from Lyman et al J Exper Zool 136 471 1957 By permission of the Journal of Experimental Zoology



Figure 10

Radiopaque material injected into heart of normal anesthetized hamster X ray taken 2 seconds after start of injection Note complete circulation of material (From Lyman and Chatfield [19])

in heart rate accompanied by a decrease in peripheral resistance [10] The exact sequence of events is difficult to time but respiratory rate and oxygen consumption rise before a detectable rise in body temperature In the animals examined to date the heart rate increases for several minutes before the blood pressure begins to rise (fig 9) Once arousal is well under way, the rise in blood pressure is rapid and is accompanied by an ever increasing metabolic rate Shivering which at first can only be determined electromyographically soon becomes so gross that the whole anterior portion of the animal shakes with the effort

It is characteristic of waking hibernators that the anterior of the animal warms rapidly while the posterior remains near the temperature of deep hibernation (see fig 9) This interesting economy of effort is caused by a circulatory control that appears to be an exaggeration of an ability found in nonhibernating mammals The rat rewarming from hypothermia shows some of the same capabilities but not to such a refined extent [18] Injection of radiopaque material into the left ventricle of hamsters arousing from hibernation has demonstrated that circulation to the posterior part of the body is inhibited and it is reasonable to assume that this is accomplished by differential vasoconstriction so that the circulation is mostly confined to the heart lungs and brain (figs 10* and 11) As arousal continues the anterior portion of the body warms to nearly 37 C before the rectal temperature changes markedly The rectal temperature then rises rapidly and within a few minutes the body temperature of the whole animal has returned to the condition found during the normal active state While the anterior part of the ground squirrel is warming the mean aortic blood pressure continues to rise but once the posterior starts to warm the blood pressure either drops or remains level (see fig 9) It seems probable that the heart is able to develop and maintain a very high blood pressure when the blood flow is restricted but that when the whole circulation is dilated it cannot compensate for the decrease in total peripheral resistance The importance of differential vasoconstriction during the warming process can be demonstrated by intra-arterial

*Figure 10 reproduced from Lyman and Chatfield J Exper Zool 114 491 1950 [19] By permission of the Journal of Experimental Zoology



Figure 11

Radiopaque material injected into heart of hamster waking from hibernation X ray taken 4 seconds after injection Note lack of circulation to the posterior

injection of norepinephrine during the waking process If such an injection is carried out while the rectal temperature is rising rapidly the temperature ceases to rise for several minutes and the blood pressure increases A series of such injections at intervals will cause the rectal temperature to rise in a step wise fashion [10]

The arousal from hibernation involves a great metabolic effort and it has been calculated that as much energy is spent in one arousal as in 10 days of hibernation Oxygen consumption rises rapidly as the anterior of the animal warms and reaches a peak at about the time the temperature of the anterior body attains 37 C At the high point of metabolic effort oxygen consumption is at least as great as in an active animal under conditions of maximum stress from exercise The heart rate at the peak of metabolic effort is often 100 times as fast as the slow rate of hibernation The whole animal seems geared to warm from its low body temperature in the least possible time and the heart beating at a rapid rate against a high head of pressure may be an inefficient pump but must contribute significantly as a heat source

If one can generalize about the hibernating cycle by combining the observations on various species of hibernators a fairly complete picture may be drawn The animal in hibernation exerts a minimum metabolic effort conducive to life deriving energy from stored fat as evidenced by a respiratory quotient of 0 7 Vasoconstriction maintains a livable mean blood pressure and other homeostatic mechanisms function efficiently enough to permit existence However the hibernating animal is always poised for arousal and even as slight a stimulus as a quick puff of air can sometimes start the waking Once started arousal usually is carried to completion In arousal the decrease in peripheral resistance is caused by a vasodilation of the anterior portion of the body while the posterior remains vasoconstricted Shivering and respiratory movements contribute most of the heat to the waking process though the totally curarized hamster can warm slowly from hibernation presumably by using chemically engendered heat [19] The main source of energy appears to be carbohydrate for the glycogen of liver and muscle is greatly depleted during arousal We have suggested that arousal is mediated and driven by a mass discharge from those somatic and sympathetic centers in the central nervous system that control temperature regulation in

he active homeothermic mammal.[3] Although ubcortical electrical exploration of the brain f the waking hamster has failed to implicate he hypothalamus in the early part of rousal,[1] there can be no question that the vhole process is under precise control and hat the autonomic system must play a part n this regulation.

The homeostasis that is typical of hiberna tion is maintained at body temperatures which are usually lethal to animals that cannot hibernate, and it seems to be typical of hiber nators that their organ systems can function at very low temperatures. Thus the peripheral nerve of the Norway rat ceases to function at 9 to 10 C, while that of the hamster will conduct at temperatures as low as 2 C (fig 12*). The hearts of most animals that do not hibernate stop between 16 and 10 C, while the hearts of some animals that hiber nate will continue an organized beat at −1 C. At slightly below this temperature the blood itself would freeze. If the heart rate is plotted against temperature in the perfused isolated heart of a nonhibernating mammal, the graph is nearly linear, and the temperature at which the heart will stop can be predicted with some accuracy by extrapolating the slope of the temperature rate curve at high temperatures. Dawe and Morrison[3] were the first to point out that this is not the case in animals that hibernate, for there is a break in the tem perature rate curve at about 15 C, and the hibernator's heart continues to beat at lower temperatures than would be predicted. The hearts of nonhibernating species that are phylogenetically closely related to species that do hibernate show no particular resistance to cold, yet the hearts of all species of hiber nators that have been tested to date are resistant to cold even though they may be only remotely related phylogenetically (fig 13). This suggests that the ability to hiber nate may have been developed separately among various species of mammals and the ability to function at low temperatures is a

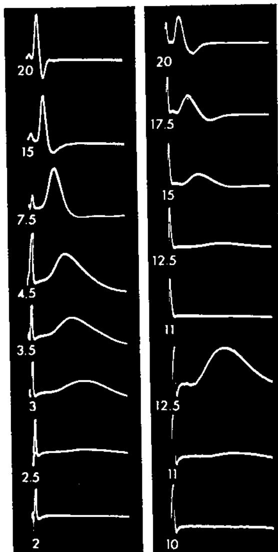


Figure 12

Effect of temperature (C) on the action potential of the isolated tibial nerve of hamster (left) and rat (right). (From Chatfield et al.[22])

result of the development of the hibernating habit.[4] For those who consider hibernation a primitive characteristic, it is of some inter est that the most primitive living rodent, the mountain beaver (*Aplodontia*), possesses a heart that is as sensitive to low temperatures as one of the most phylogenetically advanced rodents, the Norway rat (see fig 13).

The Physiologic Thermostat

If one grants that the hibernators as a group possess organs and tissues that function

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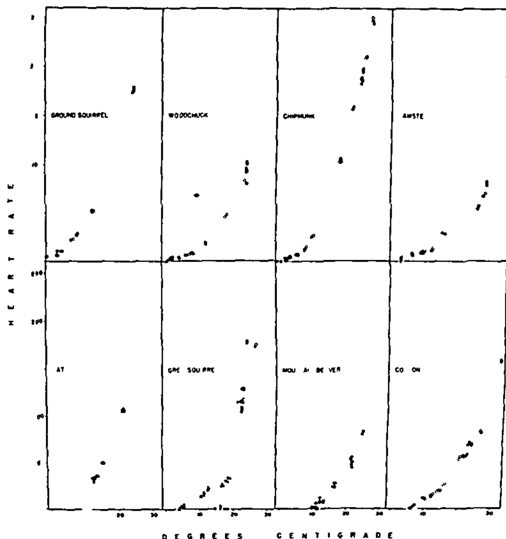


Figure 13

Effect of temperature on the rate of isolated hearts of hibernators (top four graphs) and non hibernators (bottom four graphs) Closed circles=first cooling open circles=rewarming Triangles on graph of grey squirrel indicate ectopic ventricular beats only[24]

at temperatures lethal to nonhibernators it is not hard to imagine that the hibernating mammal could keep in a steady state by re acting slowly albeit effectively to internal or external changes The problem however is not simply one of cells and tissues that can function at unusually low temperatures When exposed to extreme cold, an active hi bernator resists chilling by pilo erection shiv ering and the other methods of heat genera tion and conservation that are typical of mammals as a whole If the cold is over powering body temperature drops and the hibernator enters a hypothermic state In hypothermia a hibernator will live longer and at a lower temperature than a mammal of the same size that is incapable of hiberna tion However it cannot rewarm from near freezing temperatures without exogenous heat, and if left in hypothermia it will die within 24 hours

We are thus left with the concept that hi bernators have some way of turning down or resetting their ' physiologic thermostat ' Be cause of this ability, they have a specialization of temperature control that is unique to them

alone among the vast array of mammals as a Class The 'resetting must involve changes in both the somatic and autonomic nervous systems but the precise nature of these changes is largely a mystery We know that the changes must be coordinated and that every change must be reversible so that it is not likely that some sort of endogenous metabolic depressant plays a key role in the process It may be that hibernation is an exaggerated form of sleep If so it is a big enough problem to keep us busy for some time

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IV Contractile Proteins

Chairman Ludwig W Eichna, M D

Introduction

By LUDWIG W EICHNA M D

THIS MORNING S PROGRAM will inquire into 2 aspects of heart muscle The first of these is morphogenesis what is known about the way muscle cells grow and develop singly in culture in vitro and how the various components of the heart develop in vivo accumulate learn their positions within the organ and differentiate in the embryo to produce the final structure

The second aspect will deal with contractile protein The function of muscle is to contract and this unique biologic action is now universally considered to be brought about by the contractile proteins myosin and actin The second half of the program will consider these 2 contractile proteins derived from failing and nonfailing hearts of animals and man It will consider their biochemical and biophysical properties and their relationship to the genesis of congestive heart failure particularly as it is seen in man

Without further ado I should like to call on Dr Irwin R Konigsberg who will consider ' Some Aspects of Myogenesis in Vitro '

From the Department of Medicine State University of New York Downstate Medical Center Brooklyn New York

The Size of the Heart

it is not absolutely true that people having large hearts are cowards and on the other hand people having small hearts courageous Avicenna expresses himself well by saying that in those who have large hearts and are cowards the size of the heart is out of proportion to themselves and that such people ought to be called true cowards with their large hearts Thus when courageous persons have small hearts they are really courageous However there might be courageous persons with hearts of the right and proportioned size —R Eriksson *Andreas Vesalius First Public Anatomy at Bologna 1540 An Eyewitness Report* Uppsala and Stockholm Almqvist & Wiksells Boktryckeri Ab 1959 p 237

Some Aspects of Myogenesis in Vitro

By IRWIN R. KONIGSBERG, PH.D.

Suspensions of embryonic chick leg muscle cells have been employed to establish replicate monolayer cultures. Such cultures grow rapidly to form a confluent layer of cells. Despite culture conditions generally assumed to be dedifferentiative, a high degree of differentiation is attained in terms of the development of cross-striated myofibrils and contractility. To evaluate the possible role of in situ nuclear replication in the development of multinuclearity in muscle cells, an inhibitor of deoxyribonucleic acid (DNA) synthesis, methyl bis (beta chloroethylamine) (nitrogen mustard), was employed. Treatment with nitrogen mustard at concentration levels that profoundly inhibit DNA synthesis does not block the formation of multinuclear cells. On the basis of the pattern of nuclear enlargement after nitrogen mustard treatment, the cytologic picture of treated cultures is interpreted as indicating that the nuclei of only mononucleate cells are normally capable of proliferation. An absence of proliferative activity in the nuclei of multinuclear cells suggests that myoblast proliferation is self-limiting in this system and may explain in part the high degree of differentiation attained in monolayer culture.

IN DEVELOPMENTAL BIOLOGY, as in the biologic sciences generally, a significant factor in the development of an area of research is often the choice of the most suitable system. The system should permit ready manipulation of its components, rigid control of environmental factors, and accurate reproducibility. Tissue and organ culture technics have in the past served admirably in approaching these goals.[1] The development of satisfactory technics for preparing cell suspensions from organized tissues,[3] and for cultivating such cells on a glass substratum[4] offers a degree of precision difficult to equal.

Although tissue disaggregation has been applied fruitfully to the problem of cell affinities and the factors controlling the reorganization of tissue architecture,[5-10] the extension of the technic to the cultivation of dispersed cells has not been widely applied to developmental problems.[11-1] This is understandable in view of the prevailing opinion which holds that cellular differentiation depends on cell density.[18] With respect to this viewpoint, cell culture technics may present an opportunity to explore the nature of such a dependence.

The present studies deal with the use of monolayer cultures of cell suspensions to examine cellular differentiation in embryonic muscle cells. Embryonic skeletal muscle cells grow in cell culture and, when confluency is approached, differentiate into elongated multinuclear cells in which the development of typical cross-striated myofibrils occurs progressively and which acquire the ability to contract vigorously.[19]

Culture Methods

The sparsity of extracellular connective tissue fibrils and the relatively greater extracellular space of embryonic tissues render them more readily dissociable by brief treatment with dilute trypsin than adult tissue. Leg muscle from 11 to 12 day old chick embryos can be disaggregated by a 10 minute incubation at 37 C in 0.05 per cent crude trypsin. Tryptic action is stopped by diluting the incubation mixture with an equal volume of cold complete growth medium. Any undigested tissue residue is removed by successive filtration through gauze and 200 mesh bolting silk. The filtered cell suspension is centrifuged at low speed (1,000 r.p.m.), the supernatant decanted, and the cells are resuspended in fresh growth medium. The cell suspension is then counted in an ordinary hemocytometer and the

From the Gerontology Branch, National Heart Institute, National Institutes of Health, U. S. Public Health Service, Department of Health, Education and Welfare, Bethesda, and the Baltimore City Hospitals, Baltimore, Maryland.

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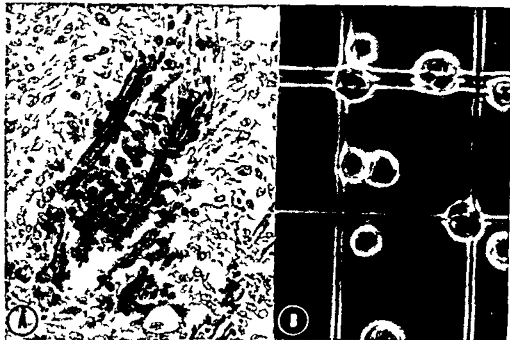


Figure 1

A Section through leg muscle of 12 day chick embryo fixed in Allen s B15 stained with hematoxylin and eosin Note the two prominent multinuclear cells in the center of the field (× 210) B Cell suspension of embryonic leg muscle cells photographed in the hemocytometer chamber (Phase contrast × 500)

desired aliquots are delivered to an appropriate culture vessel—for most purposes a 5 cm diameter Petri plate

The Initial Inoculum

One of the more disagreeable aspects of differentiation from the experimenter s view point is its lack of synchrony Thus in chick leg muscle at 12 days of incubation, although there are large numbers of undifferentiated mononucleated cells many of the cells are further advanced These comprise cells with varying degrees of multinuclearity, as well as some cells that are obviously cross striated (fig 1) Careful examination of the cell sus pensions however indicates that they consist principally of mononucleated cells Table 1 gives the distribution of nuclei per cell in 4 suspensions smeared, fixed and stained im mediately after preparation The fate of the larger cells present in the tissue is obscure They may be damaged during preparation filtered out with the undigested residue or as has been suggested by Rinaldini,[14] broken up into viable subunits as a consequence of trypsin treatment The lability of the multi nuclear condition has in fact been observed in culture [0-]

Differentiation

The conditions of cell culture are generally assumed to favor neither differentiation nor the retention of differentiative character This is apparently not true for embryonic muscle cells using the technics described above Twenty four hours after the cell suspension is pipetted into the Petri plates the cells settle out and attach to and flatten against the glass substratum The culture presents the appearance of a typical culture of 'fibro blast like cells Although the cells increase in number they remain fibroblast-like until confluency is approached At this time large numbers of extremely long multinuclear rib bon like cells appear which crisscross through out the culture (fig 2) Using an initial inoculum of 5 × 10 cells per 5 cm Petri plate this process of multinuclear cell for

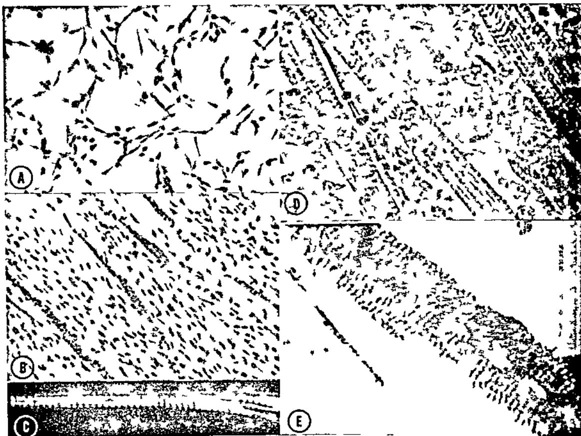


Figure 2

Developmental sequence in monolayer cultures of embryonic chick muscle cells A *Cultured cells 2 days after plating present typical fibroblast like appearance (Calcium formol fixation Ehrlich s hematoxylyin × 63)* B *Multinuclear ribbon like' cells that have formed in a sister culture (of* A) *incubated for 2 additional days Note the increased cytoplasmic basophilia of the multinuclear cells and the degree of crowding of their nuclei (Fixation staining and magnification as in* A) C *Cross striated myofibrils in a multinucleated muscle cell in 7 day old culture (Polarizing optics × 400)* D *Myofibrillar pattern in a glycerol extracted 10 day old culture (Phase contrast optics × 400)* E *Distribution of reduced tetrazolium (Nitro BT) using DPN H as a substrate in a multinuclear muscle cell of an 8 day culture The regular pattern of deposition mimics the localization of mitochondria adjacent to the I bands Note the central row of nuclei visualized by the absence of diforma an granules (Fresh tissue 15 min histochemical incubation postfixation in cold calcium formol × 400)*

mation occurs between the third and fourth day of culture In addition to their multinuclearity 2 other cytologic features distinguish the primitive muscle cells from the mononucleated cells in culture at this time

1 The cytoplasm of the multinuclear cell is extremely basophilic Similar increases in basophilia of myotubes (multinuclear muscle cells with centrally located nuclei often contiguous) have been observed in vivo and demonstrated to be due to the accumulation of ribonucleic acid [3, 4]

2 The presence of large numbers of mitochondria in the cytoplasm of the multinuclear cell [5] can be demonstrated histochemically by tetrazolium reduction using either succinate [6] DPN H or TPN H as a substrate Although reduced tetrazolium can be localized in mono

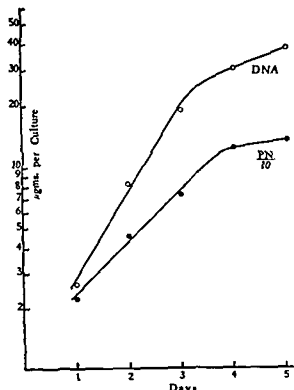


Figure 3

Semilogarithmic plot of the accumulation of deoxyribonucleic acid (DNA) open circles and protein nitrogen (PN) closed circles, per culture PN values are divided by a constant factor (10) to permit visual comparison of slopes

nucleated cells it is extremely sparse except in the macrophages

Sometime between the seventh and tenth day of culture spontaneous contractions of the multinuclear cells can be observed Spontaneous contraction of skeletal muscle cells in culture was first described by Lewis [7]

Although cross striation is not strikingly apparent using routine hematoxylin staining or phase contrast microscopy the pattern can be clearly observed *with polarizing optics* or under phase contrast microscopy after extraction of the cultures with cold 50 per cent glycerol Glycerol extraction permits detection of cross-striations between the sixth to seventh day of culture Although at first the pattern can be observed in only short lengths of a few cells with time the extent of the pattern and the number of cells in which it can be observed increase Under polarized

Table 1

Distribution of Nuclei per Cell in Cell Suspensions Prepared from 12 Day Embryonic Chick Leg Muscle

	Number of nuclei per cell				Total—all classes
	One	Two	Three	Four	
	172	24	4	1	201
	9[illegible]	11	1	0	104
	131	3[illegible]	4	1	168
	181	3[illegible]	[illegible]	1	219
Totals	576	10	11	3	69[illegible]
%	83 2	14	1 6	0 4	

light longitudinal birefringent fibrils can be detected even before cross striation can be observed Similar fibrils are observed in hematoxylin stained preparations As Holtzer has pointed out it is difficult to know whether such fibrils are in fact unstriated or have a striated pattern below the limits of resolution of the technics employed [8]

The cross striated pattern is demonstrable by still a third technic The mitochondria of striated muscle are arranged adjacent to a particular band (*I band in skeletal muscle* A band in cardiac muscle) [9] When histochemical tests for either DPN H or TPN H—cytochrome c reductases are applied the diformazan deposits are found adjacent to the I bands as with adult muscle [9,30] registering the pattern of cross striations by the localization of mitochondria (see fig 2)

The progressive nature and degree of differentiation observed in these cultures indicate that dispersed cell culturing per se is not necessarily incompatible with differentiation When such incompatibility is observed, any of several possible factors may be responsible Among these are (1) enhanced diffusion coupled with an inadequate medium (2) unrestricted proliferation that might lead to dilution of a developmentally significant cellular component (3) karyotypic changes and (4) overgrowth by an altered cell or one of different competence (i e fibroblast)

Excessive cell proliferation whatever the ultimate mechanism has frequently been implicated in the loss either permanent or transitory of differentiative expression An as-

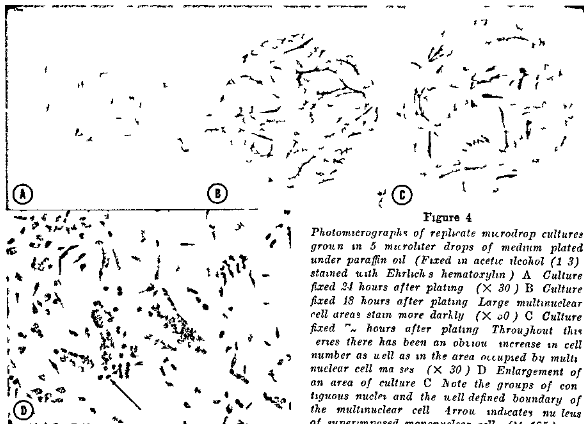


Figure 4

Photomicrographs of replicate microdrop cultures grown in 5 microliter drops of medium plated under paraffin oil (Fixed in acetic alcohol (1:3) stained with Ehrlich's hematoxylin.) A. Culture fixed 24 hours after plating (× 30.) B. Culture fixed 48 hours after plating. Large multinuclear cell areas stain more darkly (× 30.) C. Culture fixed 72 hours after plating. Throughout this series there has been an obvious increase in cell number as well as in the area occupied by multinuclear cell masses (× 30.) D. Enlargement of an area of culture C. Note the groups of contiguous nuclei and the well-defined boundary of the multinuclear cell. Arrow indicates nucleus of superimposed mononuclear cell (× 185.)

sumed antagonism between proliferation and differentiation has long received theoretical attention, at least from developmental biologists. Indeed, one of the characteristics of organ culture that is commonly employed to study differentiation is reduced proliferation.

An examination of the parameters of culture growth was undertaken to evaluate the possibility of such an antagonism in the system under examination.

Biochemical Parameters of Culture Growth

Overall proliferation has been measured by the daily increment of DNA per culture. As an index of cell size as well as cell number the protein nitrogen (PN) content of cultures has been followed. The relative growth rate* of DNA (0.0416) agrees well with the maximum rate reported by Harris[31] for the increase in cell number of embryonic skeletal muscle cells despite the differences in media and technics used. Figure 3 is a semilogarithmic plot of DNA and PN changes per culture for the first 5 days of the culture period starting from an inoculum of 5×10 cells. Logarithmic increase of both DNA and PN occurs for the first 3 days. PN accumulates during this period at a slower rate than DNA, the relative growth rates being 0.0416 for DNA and 0.0248 for PN. Whether the resultant decrease in PN per DNA indicates a progressive decrease in cell size or differential growth of the various cell types in the culture population is not known.

With an initial inoculum of 5×10^5 cells a break occurs in the rate of accumulation of both DNA and PN between the third and fourth day of culture. The appearance of multinuclear ribbons immediately precedes this break. After the break, DNA and PN continue to accumulate but at a much slower rate.

*$k = \frac{2.303\ (\log N_2 - \log N_1)}{T_2 - T_1}$

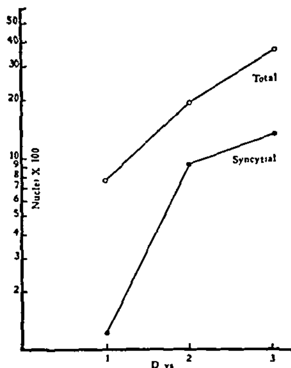


Figure 5

Semilogarithmic plot of nuclear number with time in the total population open circle and in syncytial masses closed circle

These results extend the qualitative observation that an increase in cell number occurs during the initial period of cultivation They indicate moreover, that during this period proliferation is rapid the population doubling every 24 hours Although a break in the rate of accumulation of both DNA and PN is temporally associated with the period of multinuclear cell formation any attempt at present to assess the extent to which these events are causally related would be premature

These data are an indication of overall growth however they do not of course permit a distinction between the contributions made by the various cell types in heterologous populations such as these

Nuclear Counts

Nuclear counts were made in order to compare the rate of development of multinuclearity with the rate of cell proliferation Reduction of the population size to magnitudes that would permit counting of nuclei was achieved by depositing measured microdrops (5 μL volumes) of cell suspension on coverslips submerged under paraffin oil Paraffin oil has been employed successfully in microdissection to prevent evaporation of the medium while permitting gas exchange[3]

At 24 hour intervals after depositing the microdrops cultures were fixed stained and counted Figure 4 consists of photomicrographs of such cultures fixed at 24 48 and 72 hours after plating Grossly it is apparent that the population has increased throughout the period and that the area occupied by multinuclear cells (dark staining cytoplasm) has increased at least between the first and second day Identification of syncytial nuclei is facilitated by the fact that the majority of the nuclei within syncytia are at this stage contiguous Additional criteria are the marked basophilia of the cytoplasm of the multinuclear cells and the more regular spherical shape of syncytial nuclei The occasional superimposition of a mononuclear cell over a multinuclear cell can be readily detected by differences in focal plane as well as by tracing cell boundaries (see arrow fig 4)

In figure 5 values for syncytial and total nuclear counts are plotted semilogarithmically Both sets of data have been plotted similarly for purposes of comparison The assumption is made that the increase in nuclear number is an exponential function an assumption probably applicable to mononucleated cells on theoretical grounds but of perhaps dubious validity with respect to multinucleated cells

The relative growth rate of the total nuclear population (0 0393) is in good agreement with the relative rate of DNA accumulation (0 0416) The growth rate of syncytial nuclei however is twice (2 2×) the rate of increase of the total population If the assumption were made that multinuclear cells represent a discrete and separate population of cells in which increase in nuclear number is the result of nuclear replication in situ the difference in rates of increase of the 2 nuclear populations would become greater still

If we consider increases in mononucleated

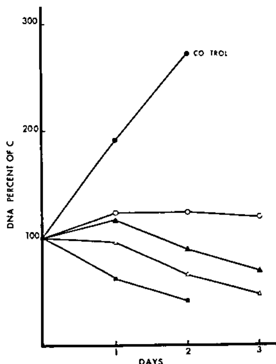


Figure 6

Deoxyribonucleic acid per culture expressed as percentages of the control level (C) at the time of treatment with nitrogen mustard Control closed circle 0.52 × 10⁻⁷ M open circle 1 56 × 10⁻⁷ M closed triangle 2 6 × 10⁻⁷ M open triangle 5.2 × 10⁻⁷ M closed square

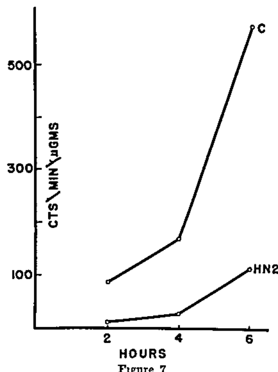


Figure 7

Specific activity of DNA thymine in control (C) and nitrogen mustard-treated (1 56 × 10⁻⁷ M) cultures (HN2) during the period from 20 to 26 hours after treatment

cells alone a relative growth rate of 0 0197 is found which is less than one fifth of the rate of increase in syncytial nuclei

Nuclear counts demonstrate in addition that at some stage nuclear division has occurred in the population of prospective myogenic cells Figure 5 indicates that by the third day of microdrop culture the number of syncytial nuclei is almost twice the total number of nuclei present at 24 hours Even assuming that all of the nuclei present at 24 hours are of myogenic cells an assumption not supported by the multiplicity of cell types observed in culture this is a clear indication of proliferative activity on the part of the myogenic elements

On the evidence currently available it seems most likely that this proliferation occurred before the myogenic cells became multinuclear

Mitotic figures are rarely if ever observed in multinuclear muscle cells [1] In the extensive cultured material used in these studies they have never been observed This fact has led to the postulation of 2 alternative mechanisms to explain the development of multinuclearity One view holds that nuclear replication does occur but by a process of direct splitting of the nuclei Support for this hypothesis is based largely upon the interpretation placed on cytologic preparations Various deformations of syncytial nuclei have been described that suggest incipient or recent splitting [33-3] Alternatively it has been postulated that multinuclearity results from successive fusion of mononucleated cells [3 25 36 37]

The Dissociation of DNA Synthesis from the Development of Multinuclearity

If multinuclearity is a product of nuclear replication it would be reasonable to assume the involvement of DNA synthesis If division

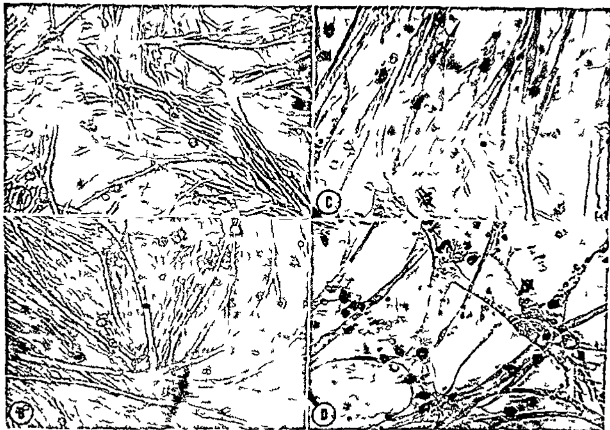


Figure 8

Photomicrographs of living cultures on 3 successive days following treatment with the highest concentration (5.2 × 10⁻⁷ M) of nitrogen mustard investigated A *Control culture at the time of application of nitrogen mustard to the experimental group Multinuclear cell formation has been initiated (note relatively small multinuclear cell)* B *Culture 24 hours after treatment with nitrogen mustard Multinuclear cell formation has continued producing typical elongated broad ribbon like cells* C *Mustard treated culture 48 hours after treatment By this time a marked decrease in the number of mononucleated cells has occurred No such loss of mononucleated cells occurs in control cultures Note dark presumably necrotic crenated cells* D *Mustard treated culture 72 hours after treatment The culture is primarily a network of multinuclear cells with bare interstices normally occupied by mononucleated cells*

occurred without DNA synthesis the distribution of values for DNA per nucleus within muscle cells should be weighted toward values lower than the diploid value However the data of Lash et al indicate that no such skewness exists [3]

To test independently the premise that multinuclearity is a product of nuclear replication an inhibitor of DNA synthesis nitrogen mustard [methyl bis(beta chloroethyl)amine] was employed [15] Solutions of nitrogen mustard were applied to cultures prior to massive formation of multinuclear cells The effects of the inhibitor on DNA accumulation and the incorporation of C^{14} formate into DNA thymine are graphed in figures 6 and 7 respectively DNA accumulation is profoundly affected even at the lowest dosage tested and the rate of incorporation into DNA thymine is reduced to less than 15 per cent of the control level by this treatment

Despite these profound effects on DNA synthesis treatment with even the highest concentration of nitrogen mustard used (5.2 ×

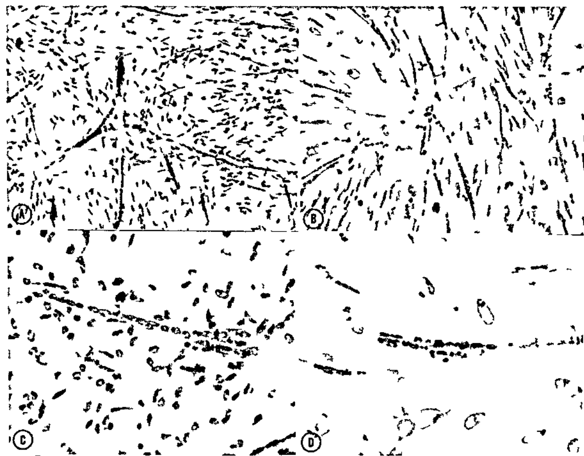


Figure 9

Control and nitrogen mustard–treated cultures of cell suspensions concentrated and replated 24 hours after treatment (1 56 × 10⁻⁷ M) Cultures were fixed in 10 per cent neutral formalin and stained with Harris hematoxylin 24 hours after replating A *Control culture in which multinuclear cell formation has occurred (× 210)* B *Culture of nitrogen mustard–treated cells Despite treatment multinuclear cell formation has occurred (linear arrangement of nuclei in more basophilic cytoplasm) The giant nuclei of treated mononuclear cells can be seen between the multinuclear cells whose nuclei are closer in size to the nuclei of control cultures (× 210)* C *Control culture at higher magnification Note the relatively uniform size of the plural nuclei in the multinuclear cell (× 495)* D *Multinuclear cell of treated culture at higher magnification Nuclear size is more variable than in the control multinuclear cell* (C) *Arrow indicates the presence of nuclear fragments or micronuclei (× 49)*

10^{-7} M) did not inhibit the development of multinuclearity (fig 8) Grossly there did not appear to be any fewer multinuclear ribbons in nitrogen mustard–treated cultures The mediation of any lag in the effect of nitrogen mustard was ruled out by testing the ability of treated cells to form multinuclear myotubes during the second day after treatment In these experiments the attainment of multinuclearity during the 24 hour incubation period after mustard treatment was circumvented by using sparsely seeded cultures To test the capacity to form multinuclear cells these cultures were then trypsinized the cell suspension concentrated and denser cultures set up in smaller vessels

Again nitrogen mustard treatment did not prevent the development of multinuclearity (fig 9) Cytologically the nitrogen mustard-treated cultures present further evidence which suggests that the nuclei of the multinuclear cells are normally nonproliferative

In his pioneering studies on the developmental effects of nitrogen mustard Bodenstein demonstrated that extreme nuclear enlargement and giant cell formation were commonly observed after mustard treatment [38, 39] The effects occurred only in those cells that would normally have been described as proliferative This was most clear cut in the case of the eye of the amphibian larva where a sharp demarcation exists between the proliferative zone and the zone of postmitotic differentiating cells

A similar situation exists in nitrogen mustard-treated muscle cultures Here the cells that exhibit marked nuclear enlargement are the mononucleated fibroblast like cells The nuclei of the multinuclear cells that form after treatment are closer to normal size However the effect of nitrogen mustard treatment is evident in these nuclei also by virtue of the wider variation in nuclear size than is normally seen (compare figs 9C and 9D) and the presence in both cell types of nuclear fragments or micronuclei

The analogy suggested is that the fibroblast like cells correspond to the cells of the proliferative zone in Bodenstein s work, while the multinuclear cells correspond to the postmitotic differentiating cells

The results of the studies with nitrogen mustard indicate that nuclear proliferation plays no significant role in the development of multinuclearity in the system under study and are compatible with the conclusions drawn from studies representing a variety of approaches

Thus microspectrophotometric analysis of regenerating mouse muscle indicates an essentially unimodal (diploid) distribution curve of DNA per nucleus in regenerating myotubes [3] Myoblast fusion has been observed[40] and recorded microcinematographically, [41] It has also been reported that interordinal chimera form in mixed cultures of mouse and chick myoblasts [42]

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Differentiation of the Atrioventricular Conducting System of the Heart

By ROBERT L DEHAAN PH D

The structure and function of the atrioventricular conducting system of the heart and its relationship to the myocardium are examined from a developmental point of view On the basis of information derived from electron micrographic electrophysiologic and developmental studies of heart tissue it is concluded that (1) The idea of the syncytial nature of the heart lacks a sound anatomic basis (2) Cytodifferentiation during embryonic cardiogenesis results in the development of at least 2 distinct populations of cells those comprising the bulk of the myocardium and a second type the specialized cells of the conductive tissue which differs in histology biochemistry and physiology (3) The common view of the myocardium as a spontaneously active tissue may require revision since several lines of evidence appear to indicate that myocardial cells are quiescent until stimulated by an extrinsic source Under normal circumstances this stimulus source is the conductive tissue

THE DEVELOPMENTAL physiologist is interested not only in the changing functions and interrelationships of organs and tissues in the embryo he is also concerned with the application of information and concepts obtained from the fields of developmental biology to problems of adult physiology The problem of the structure and function of the atrioventricular conducting system of the heart is particularly interesting when examined from a developmental point of view

In the early embryonic heart the endocardium and epicardium are separated by a thick gelatinous layer the cardiac jelly [1] Distributed through this matrix are mesenchymal cardiac myoblasts from which the bulk of the myocardium and presumably the conductive tissue will form As the myoblasts begin to differentiate taking on the characteristics typical of heart muscle the conducting tissue becomes progressively more easily distinguishable This divergence may be interpreted in two ways One is that it results from the simultaneous development of the conductive system and myocardium along dissimilar paths of differentiation It is often stated however that conductive tissue is only "specialized myocardium in which certain properties for example conductivity and spontaneity are exaggerated [2] Authors taking this position usually note that these properties are embryonic in character Thus the second possibility is implied that the two tissues are basically similar during embryogenesis differentiating along the same route but at different rates At any given stage of development the conductive system should then be less highly differentiated than the surrounding myocardium but should not exhibit any qualitatively different characteristics

We find ourselves concerned then with the degree of difference between myocardium and conductive tissue Are fibers of these two types characterized by distinct histologic cytologic and biochemical properties? Do such differences underlie clear cut physiologic differences of more than a quantitative nature? Do myocardial and conductive fibers in fact constitute two different tissues?

I shall examine these questions by drawing upon the literature concerning the physiology and development of the heart of various mammals and of the chick embryo Numerous investigators have noted the basic similarities in cardiac development of these forms

Histodifferentiation

During the early period of cardiogenesis curvature and differential growth change the heart from a primitive straight tube to a complex S shaped organ in which the four adult chambers are clearly represented (fig

From the Department of Embryology Carnegie Institution of Washington Baltimore Maryland

1) The beat is initiated at the posterior end of the heart As the primordial atrioventricular (A V) atrial and sinoatrial (S A) regions progressively form each of them in turn takes over the pacemaker function initiating the beat for the entire heart At these early stages the primordial atrial and ventricular muscle are in direct continuity around the entire circumference of the A V canal A stimulus arising in the sinoatrial region should be able to spread throughout the heart freely to all areas That this is true has been demonstrated by Patten[3] who cut away all of the tissue around the A V canal of a 4 day chick heart except for a narrow connecting strand (fig 2) * This strand then served as an artificial bundle to conduct the sinoatrial rhythm to the ventricles He found that it made no difference whether the connecting strand was left at the site where the normal bundle would later develop (marked with an asterisk in fig 2) or whether the strand was left on the opposite wall of the heart

During the fifth and sixth weeks of human development endocardial and epicardial connective tissue gradually encroaches on the myocardium at the A V sulcus and invades this connecting zone The separation of the atria and ventricles is ultimately completed all the way around the sulcus as the primordial fibrous skeleton of the heart is formed However early in the sixth week of development (9 to 10 mm human embryo) a compact cluster of cells makes its appearance in the posterior wall of the A V canal toward its right side[4] This is the A V node in cellular continuity with the atrial muscle above and narrowing into the A V bundle below The bundle runs across the top of the thick interventricular septum behind and under the dorsal endocardial cushion (fig 3†) At this stage relatively little cytologic differentiation has yet occurred in either the node or

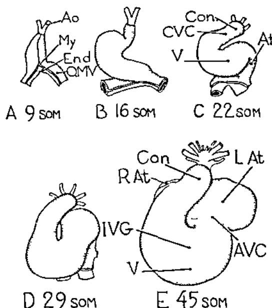


Figure 1

Early stages in cardiac morphogenesis In the chick the stages illustrated would be at about (A) 36 hours (B) 46 hours (C) 52 hours (D) 62 hours and (E) 3½ to 4 days Equivalent stages would occur during the first 4 weeks of development in the human At (L and R) left and right atrium Ao ventral aortic root A V C atrioventricular constriction Con conus arteriosus C V C conoventricular constriction End endocardial tube I V G interventricular groove O M V omphalomesenteric vein My cut edge of myocardium V ventricle (Redrawn from Patten[68])

bundle The bundle of course is not invaded by the encroaching connective tissue and remains as the single fascicle of conducting fibers connecting the atria and ventricles

Within a week or 2 the bundle branches arise and the node and bundle become clearly distinguishable from the surrounding myocardium as pale staining meshworks of cells with rounded nuclei containing scattered and poorly striated myofibrillae (fig 4) As they approach the apex of the heart both the left and right bundle branches ramify into progressively smaller groups of fibers with much interlacing and anastomosis[5,6] The cells of the branches proximally are similar histologically to those of the bundle itself which in turn resemble nodal cells As

* Figure reproduced from Patten Univ Michigan M Bull 1 1956 By permission of the Michigan University Medical Bulletin

† Figure 3 reproduced from Walls J Anat 81 93 1947 By permission of the Journal of Anatomy

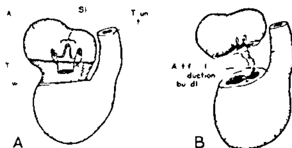


Figure 2

Heart of a 4 day chick embryo dissected to leave a connecting muscle strand between atrium and ventricle simulating an ectopic A V bundle A *Dorsal view of heart with diagonal hatching indicating the tissue to be removed* B *Same heart after dissection (From Patten[9])*

the branches ramify distally they become swollen and watery exhibiting a central juxtanuclear hyaline space peripheral poorly formed myofibrils and scattered mitochondria that is they become typical Purkinje fibers (fig 5) In the human embryo such fibers do not appear until rather late probably between the tenth and fifteenth week of development (60 to 100 mm)[4, 7] However by the end of the fetal period a complex interlacing basketwork of specialized fibers invades all parts of the ventricular musculature A reconstruction of the entire ventricular conducting system is shown in figure 6 which is a retouched photograph of a model made by Lydia DeWitt[8] It shows the A V node bundle branches and the major ramifications The fine terminal' Purkinje fibers are not shown (Throughout this paper the tissue comprising the S A and A V nodes the bundle branches and Purkinje fibers will be referred to as 'conductive tissue'')

The consequence of all this embryonic histodifferentiation is the presence of two readily distinguishable types of fibers in the adult heart of most mammals and birds Some of their respective properties are summarized in table 1 which compares certain histochemical and cytologic properties of typical myocardial conductive and embryonic heart cells

In spite of their manifest differences, we should remember that many similarities also exist between myocardium and conductive tissue Both types of fibers exhibit intercalated discs, striated myofibrils sarcosomes and other cytoplasmic inclusions characteristic of muscle Both contain antigenic combining groups of cardiac myosin[21] Moreover, it is now generally accepted that they are also physiologically similar, in that all heart tissue is characterized by the 3 functional properties contractility conductivity and spontaneity Let me quote from 2 representative sources on this matter In a recent paper on the ' Microanatomy of Muscle Walls[2] states that

> ''Cardiac muscle tissue is found only in the heart and surrounding the mouths of the great veins which enter it In some ways it resembles both voluntary and smooth muscle yet in its rhythmic unceasing activity from early embryonic life until death it stands alone (p 5.)

And in his chapter in Fulton s Physiology, Nahum calls our attention to the fact

> that all parts of the musculature of the heart are inherently capable of autogenic rhythmic discharge and that the normal sequence of excitation is made possible only because the sinoatrial node possesses this capacity in a higher degree than any other region (p 6,°)

Thus we are told that the properties of cardiac muscle distinguish it clearly from other types of muscle and also that heart muscle consists of a basically homogeneous population of fibers all having essentially the same properties showing only certain minor differences in degree in different regions

The idea of the heart as a syncytium of fibers in cytoplasmic continuity tends to foster this concept of homogeneity

Cellular versus Syncytial Structure

Early histologists considered heart muscle as cellular in structure and interpreted the intercalated discs as junctional zones of apposed cell membranes staining heavily as a result of intercellular cement substance For example Eberth [3] to whom the first detailed description of the intercalated disc is usually attributed in 1866 regarded the discs as intercellular structures not only on the basis

Table 1

Properties of Adult Myocardium Conduction Tissue and Embryonic Heart

	My d	Cnd t	Emb Hrt	References
Cytology				
Myofibrils				
Well formed densely regularly packed	+	—	—	9 10 11 12 13 14
Heavy parallel striations	+	—	—	9 10 11 12 13 14 15
Run parallel to cell axis	+	—	—	9 10 11 1 13 14 15
Prodromal striation pattern	—	+	+	9
Cytoplasmic inclusions				
Loose non striated filaments	—	+	+	9 10 11 12 14
Well formed granular endoplasmic retic	+	—	—	9 10 11 1
Mitochondria few and juxtanuclear	—	+	?	9 10 11 12 14 15
Rows or clusters of Palade granules	—	+	+	9 11 14
Numerous glycogen granules	—	+	+	9 11 15 16 17
Intercalated discs				
Interdigitating and complex	+	—	—	9 13 14
Histology				
Stain heavily with				
Hematoxylin eosin	+	—	—	15 17
Silver impregnation	+	—	?	17 18
Iron hematoxylin	+	—	—	17
Mallory trichrome	+	—	?	18
Glycogen Schiff reaction	—	+	+	16 17 19
Masson s trichrome brick red or purple gray	BR	PG	?	17 0

of their histologic appearance and their strong reaction with silver nitrate (known to stain cell membranes) but also because of the tendency of macerated heart muscle to fragment along the intercalated discs Later Ranvier [4] also described mammalian myocardium as being composed of individual rhomboidal branching cells with centrally placed nuclei

It was not until after the turn of the century that Heidenhain[25] and Godlewski[26] independently enunciated clearly the hypothesis that the heart was syncytial in nature These workers felt that the discs represented either contraction artifacts or the sites of sarcomere differentiation Perhaps the strongest proponent of the syncytial nature of adult myocardium was H E Jordan who over a period of a decade or more published observations and strenuous arguments in support of this idea [27, 8] (It is interesting to note however that even Jordan was compelled to admit to the cellular structure of Purkinje tissue and of embryonic heart)

The controversy over syncytium versus cells recurred periodically in the literature of the first half of this century as some microscopists continued to make observations corroborating the earlier cell view [9] However most investigators during this period gradually tended to accept the syncytial hypothesis and even extended it to the conductive tissue [30]

Especially important in this trend toward the idea of a cardiac syncytium were the early demonstrations of the all or none' response of heart tissue by Bowditch [31] Woodworth [32] and others It was felt that this finding could best be explained on the basis of protoplasmic continuity between all cardiac fibers During the last decade also confirmatory electrophysiologic evidence has been obtained In 1951 Curtis and Travis[33] reported that bundles of Purkinje fibers in the false tendon of the ox heart responded to electric stimulation in an all or none manner that is the demarcation potential measured between the cut end and the surface remained at con

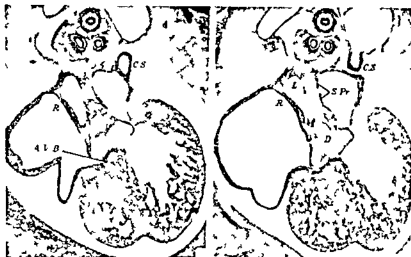


Figure 3

Section through the heart of a 10 mm human embryo Left The bundle (A V B) may be seen arising from the A V node and passing into the base of the dorsal endocardial cushion Right Continuation of the bundle down the left side of the interventricular septum P=right sinus valve L=left sinus valve, C S=coronary sinus S Pr=septum primum D=dorsal endocardial cushion (Hematoxylin eosin stain × 30) (From Walls[4])

stant amplitude with varying strengths of suprathreshold stimuli Thus the fibers appeared to behave syncytially This was further supported by measurements of specific core resistance of Purkinje fibers by Weidmann[34] He showed that although the resistance of the cylindrical membrane surrounding a fiber was of the same order as that of muscle or nerve the core resistance (i e the longitudinal internal resistance) was not appreciably higher than that of the sarcoplasm He concluded that the intercalated discs do not form a significant barrier to ionic movement along the length of the fiber

In spite of the evidence of homogeneity (and even identity) of all fibers Thomas Lewis (1925) after some 20 years of pioneering work in cardiac electrophysiology proposed his law of cardiac muscle [35] In this statement he noted that there are different kinds of heart cells those characteristic of myocardium and typical Purkinje fibers Because of the prevailing opinions this idea was not strongly favored and few references to it appear in recent decades

In the last few years, however since electron microscopy has been applied to this problem, it has been shown repeatedly and without exception that the idea of a cardiac syncytium lacks a sound anatomic basis Perhaps a dozen electron microscopists using ever more sophisticated technics have found that the intercalated disc does consist of a pair of apposed cell membranes covered with densely staining granules The myofibrils do not cross the intercalated discs nor is there any other evidence for any kind of protoplasmic continuity across the membranes Each elongate cell of a myocardial fiber is consistently observed to be surrounded by an intact plasma membrane (for recent review of evidence see [36]) This is true in both myocardium and conductive tissue [9, 12] Moreover the pattern of development of the intercalated discs in cardiac myoblasts at the sites where the myofibrils are interrupted by cell membranes also supports the idea of a cellular structure [10, 13] Thus neither in the embryo nor in the adult heart neither in myocardium of cold blooded[37] or warm blooded vertebrates nor in nodal or conductive tissue is there a satisfactory anatomic basis for the notion of syncytial structure

A Functional Syncytium?

The studies of Curtis and Travis[33] and of Weidmann[34] noted above led to the use of the concept of heart tissue as a "functional syncytium" By this it is implied that even though cardiac fibers do not in fact exhibit protoplasmic continuity under certain conditions they behave as if they did For example in small cultured fragments (50 to 100 microns in diameter) of embryonic heart tissue the membrane effects of a polarizing

current applied through one of a pair of intracellular microelectrodes can be recorded in any cell of the fragment even when the recording electrode is as much as 100 microns from that used for stimulation and visible cell boundaries separate the two[38] Moreover synchronous and identical diastolic depolarization and action potentials can be recorded from all cells of such a spontaneously beating clump

However other physiologic evidence is accumulating which suggests that individual cells or at least small regions of cardiac tissue are capable of separate activity independent of neighboring cells or regions Embryonic heart for example can be disaggregated with trypsin into small cell clusters from which transmembrane resting and action potentials can be recorded Such recordings are similar in all respects to those obtainable from cells in the intact heart[9] Thus the electrical activity of these cells is clearly unaffected by cellular dissociation Moreover in the cooled adult mammalian heart[40] or frog heart perfused with hypertonic Ringer sucrose solution[41] it is possible to record spontaneous action potentials from one fiber while a neighboring fiber is completely quiescent or fires at a different rate Similarly Cervoni West and Falk[4] recording intracellularly from rabbit atrial preparations found that when such a preparation was stimulated at 7 to 8 beats/sec atrial cells responded to every stimulus while nearby cells from the S A node responded only to every other stimulus At a time when a nodal cell was still in its refractory period a neighboring atrial cell was fully repolarized and capable of firing

Thus the weight of anatomic evidence appears to support the concept of the heart as a population of discrete cells separated transversely by intercalated discs Whatever the normal mechanism of transmission of the impulse across the discs physiologic studies show that such transmission can readily be disturbed with the result that the individual cells or contractile units can exhibit their independent nature The histologic differ-



Figure 4

Cross section of the A V bundle (B) and root of the right bundle branch (RB) in the crest of the interventricular septum (IVS) of a " day chick embryo heart (Phosphotungstic acid hematoxylin × 00)

entiation described above would further suggest that there are at least two distinct cell types that making up the bulk of the myocardium and a clearly different perhaps more embryonic type which constitutes the conductive system (Space does not permit mention here of the evidence for intermediate types[9 43]) It is evident that processes of cell differentiation leading to such histologic differences must be based upon changes at the biochemical level Is there then any evidence of biochemical or physiologic differentiation between myocardial and conductive cells?

Biochemical Differentiation

Quantitative biochemical differences between myocardial and conductive cells are many (see Schiebler[17] for review) For example conductive tissue contains more choline acetylase than does myocardium[44 4] Healthy adult myocardium is quite low in glycogen[46] whereas the rich concentration of glycogen in conductive tissue has been noted repeatedly[17] and can even be seen with in



Figure 5
Subendocardial Purkinje cells (P) compared with myocardium (M) in a newly hatched chick embryo heart (Phosphotungstic acid hematoxylin × 400.)

travital staining in the living heart[4] Its abundant supply of glycogen would suggest that conductive tisue might be highly dependent on glycolytic metabolism perhaps in lieu of a strong oxidative cycle This idea is supported by the finding that conductive tissue has a much lower succinic dehydrogenase activity than does myocardium and has a total oxygen consumption rate only one fifth as great It is also resistant to cyanide and to anoxia[19]

There are 2 enzymes or enzyme systems in conductive tissue that appear to be qualitatively absent from myocardium in most species (It is recognized that such a claim based upon negative evidence is valid only within the limits of presently available techniques) Although nonspecific cholinesterases are abundant in all heart cells Mommaerts et al,[48] were able to characterize an enzyme from the A V bundle and bundle branches of beef heart as a specific cholinesterase similar to that characteristic of nervous tissue More recently Carbonell[49] confirmed this and localized the enzyme histochemically to cells of the conduction system in hearts of the human dog cat, guinea pig rabbit rat sheep and cow Only in the rabbit were equivocal results obtained in which scattered cells in the myocardium exhibited esterase activity that was nonspecific in its substrate specificity but was inhibited by eserine (1 of the criteria for specific cholinesterases) In the other species mentioned specific cholinesterase was limited to cells of the conduction system (fig 7*)

The second enzymatic activity seen exclusively in conductive tissue is that responsible for a phenomenon termed 'aberrant lipogenesis and suggests a distinctive fat metabolism in this tissue Recently Kuwabara and Cogan[50] have shown that Purkinje tissue can synthesize sudanophilic fat when incubated in serum supplemented by oleic acid a capacity not shared by ordinary cardiac muscle This property depends upon the presence of (1) an intracellular sulfhydryl requiring enzyme system (2) serum and (3) oleic acid or sodium oleate as a substrate It is clear from their work that cells of the conductive system exhibit this enzyme whereas those of the myocardium apparently do not

It is interesting to note that many of the biochemical and metabolic characteristics distinguishing conductive tissue from myocardium are the same as those differentiating embryonic from adult heart Heart muscle of the early embryo is rich in glycogen[48 1] and highly resistant to anoxia[5] It is also low in succinoxidase[53] and cytochrome oxidase[54] activity and is relatively insensitive to cyanide poisoning[51] Conductive tissue is thus distinctly embryonic ' in sharing some of the cytologic histochemical and biochemical characteristics of primitive heart At the

*Figure 7 reproduced from Carbonell J Histochem 4 8 1956 By permission of the author and The Williams & Wilkins Co



Figure 6

The atrioventricular conducting system of the calf heart retouched to clarify its 3 dimensional aspects (Modified from DeWitt[8])

same time, however it shows definite signs of having differentiated along an independent path developing a distinctive morphology and biochemistry with at least two enzymes not shared with myocardium

Physiologic Differentiation

Does the physiology of myocardium and conductive tissue show a similar pattern that is do these 2 tissues exhibit some functions that differ only in degree and others that are totally restricted to 1 or the other?

One well known quantitive physiologic difference between myocardium and the conductive system is in conduction velocity Since the observations of Erlanger[56] it has been accepted that the conduction tissue is capable of transmitting an impulse more rapidly than myocardial muscle (with the exception of certain specific areas as at the atrionodal junction) In 1959 Draper and Mya tu[57] measured the rate of conduction through specialized fibers in false tendons and in the bundle branches at a velocity of 2 to 3 meters/second In contrast strips of parallel myocardial fibers not containing Purkinje tissue were able to conduct the stimulus at a rate of only 0 5 to 0 6 meter/second Clearly this represents a differentiation of new membrane characteristics by the specialized fibers to permit such rapid conduction since embryonic myocardium starts out with a low conduction rate comparable to that which is apparently retained by adult myocardial muscle[58]

A second difference between myocardial and conductive cells at a physiologic level lies in the configuration of their action potentials Intracellular recording from cells in various parts of the conductive system reveals action potentials that when compared with those from myocardial fibers have a

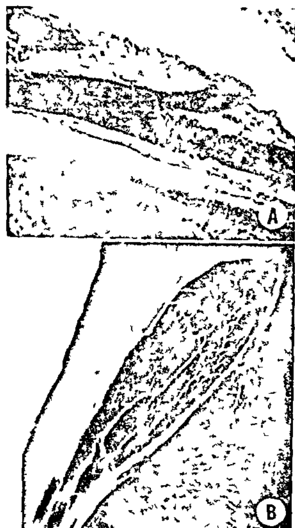


Figure 7

Specific cholinesterase activity in conduction tissue of the adult human heart. A. Subendocardial Purkinje fibers. B. A-V bundle and left branch. (From Carbonell.[9])

low amplitude with little or no overshoot, a low rising velocity of the upstroke and long duration with a rounded plateau. In contrast to the steady resting potential common to myocardial fibers, recordings from pacemaker regions also show a characteristic slow diastolic depolarization of 10 to 20 mv.[43, 59] termed the "prepotential" by Bozler.[60] The prepotential in association with pacemaker activity differentiates extremely early in caridac development, long before specialized nodal or conduction tissue is histologically distinguishable. Recently Meda and Ferroni[61] have been able to insert intracellular electrodes into hearts of chick embryos only a few hours after initiation of the beat. Even at these early stages (see fig. 1 *A* and *B*) cells showing a slow diastolic depolarization were found in the S-A region, while cells in the ventricle showed steady resting potentials similar to those from adult myocardium (fig. 8*). Thus embryonic ventricular cells even in the 13 somite chick embryo do not originate their own beat but are stimulated by cells in more posterior regions which are acting as pacemakers.

This leads us to the final physiologic property that I should like to compare between myocardium and conductive tissue: the property of spontaneity or pacemaker function. We have seen that in the embryonic heart ventricular cells do not initiate their own beat but are driven by other cells. It is commonly accepted also that in the adult heart the contraction stimulus arises in the definitive pacemaker, the S-A node, and is transmitted to all parts of the heart via the fibers of the conduction system. Yet, as noted earlier, the idea that all heart muscle is characterized by the property of spontaneity seems to pervade the thinking of most cardiac physiologists, and it is stated explicitly in textbooks and other reference sources. Evidence appears to be accumulating, however, which suggests that the ability to generate bioelectric potentials, i.e., the pacemaker function, may not be a property common in varying degrees to all heart cells but may be limited exclusively to cells that differentiate into some component of the conduction system. Myocardium, in contrast, appears to be completely quiescent until stimulated by an extrinsic source in a manner analogous with skeletal muscle.

It should be emphasized that a systematic analysis of this problem has not been carried out and decisive experiments are few. However, I should like to consider some of the

*Figure 8 reproduced from Meda and Ferroni: Experientia 15: 4[illegible], 1959.[61] By permission of Experientia.

relevant data. In 1910 Erlanger[6] showed that equal sized strips of cat atrium isolated in vitro may be spontaneous or not depending on what region of the atrial tissue is included. Strips of the left atrium frequently remained quiescent while similar areas of right atrium or of left atrium connected to the interatrial septum usually showed spontaneous activity. We now know that those areas which Erlanger found it necessary to include in a strip if it was to beat spontaneously are the regions from which pacemaker prepotentials have been recorded, namely the interatrial septum, the crista terminalis, the entrance of the superior vena cava and the S-A ring bundle.[43, 63]

Evidence that spontaneity is limited to a portion of the cells of the heart arises also from observations made on such cells in tissue culture. Cavanaugh[64] disaggregated 4 to 6 day embryonic chick hearts. She found that immediately after the cells attached to the glass substratum only 9 per cent showed spontaneous activity. Within the first day or two after the cells had spread and established extensive filopodial interconnections the proportion pulsating rose to about 50 per cent. Moreover, as interconnections were established in the culture, cells that had been quiescent or beating independently could be influenced by a dominant neighbor to contract in synchrony with it. Other examples of such pacemaker activity by embryonic cells or heart fragments exist in the literature,[8, 6, 66] and Harary and Farley[67] have recently shown similar behavior on the part of dissociated cells of young (nonembryonic) rat hearts. Thus it seems reasonable to conclude that in neither embryonic nor adult heart is the majority of cells capable of spontaneous contraction. It is recognized, however, that this conclusion may be premature when applied to the embryo in view of the apparent lability of pacemaker function in immature myocardium. Cells in the embryonic ventricle in situ do not show pacemaker prepotentials.[61] However, cell clusters from this tissue in culture beat

Figure 8

Action potentials recorded from the heart of a 42 hour (13 somite) chick embryo. Top: *Electrode inserted in the posterior end of the heart tube in the presumptive atrial region; note the diastolic depolarization of approximately 10 mv.* Bottom: *Electrode inserted in the primitive ventricle; note the greater amplitude and shorter duration of these action potentials as measured by the amplitude and time calibrations shown. (From Meda and Ferroni.[61])*

spontaneously and do exhibit slow diastolic depolarization.[38] Thus pacemaker activity in ventricular cells may be lost or suppressed during development of the intact heart but may be regained by at least some cells when isolated and subjected to culture conditions.

In adult heart, however, spontaneity does appear to reside exclusively in those cells that have differentiated into components of the conduction system. This argument has gained substantial strength from the recent observations of Draper and Mya-tu.[57] These investigators confirmed the findings of many others[68] by showing that muscle slips or papillary muscles from various mammalian hearts maintained in vitro are frequently not spontaneously active. However, they also noted that when such spontaneity is displayed it "can always be traced to the presence of Purkinje tissue in the sample" (p. 107[7]). For example, in one group of 18 ventricular muscle slips, 9 did not beat spontaneously;

yet all responded vigorously to external stimulation for many hours and showed normal resting and action potentials These muscle slips were serially sectioned for histologic examination Those that had not beat spontaneously showed no evidence of Purkinje tissue while specialized fibers were found in all those that were active Thus we may conclude that in adult heart, if conductive cells are present to initiate an impulse (and possibly a critical number is required), we see spontaneous activity Without these cells the heart muscle though healthy and completely responsive remains quiescent

Conclusions

When information obtained from studies of the structural chemical and functional properties of the A V conducting system of the adult and embryonic heart is considered different conclusions may be drawn than those arrived at by a purely physiologic approach Though not yet firmly established by decisive experiments these conclusions appear to be reasonable in view of the evidence presented They may be stated as follows (1) Cellular differentiation during embryonic development of the heart leads to the presence of 2 distinct populations of cells typical myocardial cells comprising the bulk of the musculature of the heart and the specialized cells of the conductive tissue (2) Although cells of the conductive system do exhibit certain of the properties of embryonic myocardium the two tissues in the adult may be distinguished by characteristic and unique properties at the histologic biochemical and physiologic levels of investigation (3) The differentiation of pacemaker function occurs very early in cardiac development Within hours after initiation of the beat and perhaps earlier cells of the ventricle do not originate their own beat but are stimulated by other cells functioning as pacemakers (4) In the adult heart myocardial cells appear to be completely quiescent until stimulated by an extrinsic source Under normal circumstances this stimulus source is a pacemaker cell (or cells) of the S A node, or some other part of the conduction system

Acknowledgment

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Cardiac Myosin and Congestive Heart Failure in the Dog

By Robert E Olson Ph D M D Eric Ellenbogen Ph D *
and Raja Iyengar Ph D

Chronic congestive heart failure has been produced in dogs by surgical induction of valvular disease Cardiac myosin was isolated from the normal dogs and from dogs with congestive heart failure and characterized Physicochemical properties of the cardiac myosins were determined by measurements of velocity sedimentation partial specific volume rate of diffusion limiting viscosity number light scattering behavior and ATPase activity The measurements show that normal cardiac myosin (myosin C) has a molecular weight of [illegible]5 000 whereas myosin from the failing heart (myosin F) has a molecular weight of 690 000 This change in molecular weight occurs without a marked alteration in amino acid composition and suggests that end to end trimerization of normal cardiac myosin occurs in association with congestive heart failure in the dog The e as [illegible] significant change in ATPase activity

FOR THE PAST several years our laboratory has been engaged in the systematic study of cardiac metabolism in the dog in various conditions of malnutrition endocrine imbalance [1] and surgically induced valvular heart disease Of particular interest has been the study of the contractile proteins of the heart in various states including experimental chronic congestive heart failure

This study was undertaken because of the inability of many investigators [3] including ourselves [4] to discover in either dogs or man with heart failure any evidence of a biochemical defect in the uptake of substrate from the coronary blood the oxidation of these substrates to carbon dioxide and water or in the process of oxidative phosphorylation [5] The shortening of the myofibril in the contractile process involves the interaction of at least 2 contractile proteins myosin and actin with ATP Hence in view of the above negative evidence it seemed important to study the properties of these proteins in hearts of animals in various states of cardiac compensation Since appropriate samples of cardiac muscle from human subjects are very difficult to obtain immediately after death it was necessary to study this problem in an experimental animal This report presents the results of studies of the physicochemical properties of cardiac myosin isolated from normal dogs from dogs with sodium retention after ligation of the inferior vena cava and from dogs with chronic congestive heart failure from valvular disease More detailed reports of these findings have appeared elsewhere [6, 7]

Methods

Normal mongrel dogs immunized upon arrival from the kennel and known to be in good health through observation in our animal colony over several weeks served as the normal control group for this study Dogs with inferior vena cava ligation (ICL) having marked sodium retention and ascites served as the second control group This ICL group was a particularly good control group because these animals possessed normal cardiac contractility in the presence of sodium retention and altered aldosterone metabolism [8, 9]

In the first experimental group congestive heart failure was produced in a series of animals by surgical avulsion of the tricuspid valve and stenosis of the pulmonary artery essentially by the method of Barger Roe and Richardson [10] This combination of surgical lesions which was accomplished in 2 stages at 3 week intervals produced congestive heart failure in 60 per cent of the surviving dogs with an operative mortality of less than 15 per cent These animals began to show clinical signs of congestive heart failure within 1 to 4 weeks after constriction of the pulmonary artery These signs included oliguria marked sodium retention an increase in body weight with the appearance of ascites and reduced exercise tolerance

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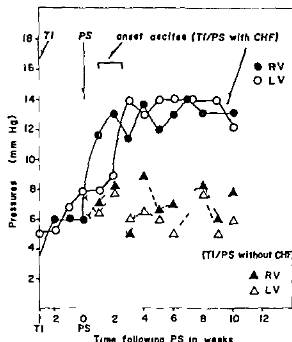


Figure 1

Ventricular end diastolic pressures in dogs operated upon to produce tricuspid insufficiency and pulmonic stenosis. The time of each operation is noted. The end diastolic pressures were measured in nonanesthetized dogs. The solid symbols are right ventricular pressures; the open circles are left ventricular pressures. The lower set of curves represents the pressures in animals that did not develop congestive heart failure.

In the second experimental group of dogs, primary left heart failure was produced by creating a mitral insufficiency followed by 1 of 2 aortic lesions. Aortic stenosis was produced by resecting the noncoronary aortic cusp and adjacent aorta with the formation of a bicuspid valve along the lines suggested by Carmella, Andersen and Oropeza.[11] A constriction in the lumen of about 60 per cent was achieved. Aortic insufficiency was induced by punching holes in the noncoronary cusp with a circular valvulotome through the aorta according to Roshe and Morrow.[1] Postoperatively the intraventricular pressures of many of these dogs were measured through the chest wall at intervals to determine the time of onset of failure. Statham gauges and a Sanborn visocardiette were employed to record the pressures. Minimal morphine sedation was used to facilitate the procedure so that the dogs were quiet but awake and responsive. All of the animals sacrificed for characterization of cardiac myosin had been in failure for at least 6 weeks as evidenced by elevated end diastolic pressures.

In order to describe the hemodynamic status of these animals prior to sacrifice, a light plane of anesthesia was induced in each animal by the intravenous administration of Nembutal–Dial Urethane ([illegible] v/v). Cardiac catheterization of the right heart and coronary sinus was accomplished, a femoral artery was cannulated and pressure measurements were made in the femoral artery, right atrium, right ventricle and pulmonary artery by means of Statham gauge transducers with either a Sanborn twin viso-cardiette or a Medical Electronics recorder. In some instances simultaneous tracings in right ventricle and left ventricle were recorded using a direct puncture of the left ventricle through the chest wall (see figure 2). Myocardial oxygen and carbon dioxide exchange and substrate extraction were measured by sampling simultaneously from the coronary sinus and the femoral artery. Coronary flow was measured by the nitrous oxide method.[13] Blood gases and substrates were measured by methods previously described and the results of studies of the metabolic activity of the failing heart are reported elsewhere.[14]

At the conclusion of the physiologic measurements the plane of anesthesia was deepened by administration of additional Nembutal to permit a thoracotomy and institution of artificial respiration. The pericardium was opened and the animal sacrificed by rapid excision of the beating heart. The organ was immediately chilled in deionized water in 1 C. Generally a ventricular ectopic rhythm persisted until the heart had been immersed in the cold water for a few seconds. After being fully chilled to 1 C the heart was dissected in a cold room to remove fat and connective and atrial tissue. Both right and left ventricular tissue were combined and minced in a meat grinder at 4 C, and the myosin was isolated by the method previously described.[6]

Cardiac myosin was isolated from dogs in the 2 control and in the 2 experimental groups and characterized by a study of velocity sedimentation, partial specific volume, rate of free diffusion, limiting viscosity number, light scattering behavior and ATPase activity. Partial specific volumes were determined by pyknometry. ATPase activity was determined at 25 C on samples dialyzing against veronal buffer (pH 8.6) in order to remove phosphate ions. The method of Gergely[15] was employed and the ATPase activity expressed as Q_p (microliters of phosphorus liberated per milligram of myosin per hour). Sedimentation velocity measurements were carried out in a Spinco Model E analytical ultracentrifuge. Solutions above 0.2 per cent protein were analyzed in the conventional manner from schlieren patterns. More dilute solutions were

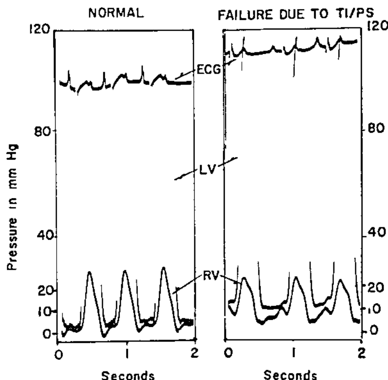


Figure 2

Tracings of right and left ventricular pressures are shown for a normal dog in the panel on the left and for a dog in congestive heart failure caused by TI/PS in the panel on the right An electrocardiogram is included in each case for reference The end diastolic pressures in both the right and left ventricles are elevated in the animal with congestive heart failure

analyzed by measurement of ultraviolet absorption using a Spinco analytrol to plot the density of the absorption bands Sedimentation runs were carried out both at 24 and 4 C no dependence of sedimentation constant upon temperature or speed of rotor was noted Diffusion constants were estimated both from boundary spreading observed in the ultracentrifuge and from free diffusion in the Spinco Model H electrophoresis apparatus Diffusion constants were calculated from schlieren patterns and from Rayleigh fringes respectively employing the method of second moments The boundary spreading coefficients were corrected for the concentration dependence of the sedimentation constant Light scattering measurements were carried out in a Brice Phoenix light scattering photometer equipped with a Brown recorder The wave length chosen was a mercury blue line (436 mμ) The refractive index increment was determined at the same wave length in a Phoenix differential refractometer and found to be 0 206 on a weight fraction basis The solutions were clarified and measurements were carried out at 0 45 90 and 135 degrees at a temperature of 15 C or less The limiting viscosity number was obtained by measurement of the viscosity of solutions of different concentrations in an Ostwald viscometer with a water time of about 180 seconds mounted kinematically in an unsilvered Dewar flask at 2 C Triplicate determinations were obtained with a maximal deviation of ± 0 3 second Further details of these method have been published [6]

Three preparations of normal cardiac myosin and the myosin obtained from the failing heart were hydrolyzed in 6N HCl and their amino acid was analyzed by the method of Moore and Stein [16, 17]

Results

Physiologic Studies

All the animals in this series with valvular disease showed generalized congestive heart failure at the time they were sacrificed for the study of cardiac myosin Both the normal animals and the controls with inferior vena cava ligation showed no evidence of congestive heart failure

In several of the animals operated upon the development of failure was followed by measurements of end diastolic pressures in the right and left ventricles Results of a typical series of animals is presented in figure 1 After production of tricuspid insufficiency end diastolic filling pressure in the right heart was elevated from 2 to approxi

Table 1

Hemodynamic Findings in Control and Experimental Dogs

Condition	No	Body weight Kg	Ascites L.	Cardiac index L./min/M²	Coronary flow Ml/100 Gm/min	Oxygen usage Ml/100 Gm/min
		19 9	—	2 59	87	10 8
Normal	1*	±1 1	—	±0 18	±6	±1 0
		16 9	7 0	1 68	115	1[illegible] 3
ICL	5	±1 8	±[illegible] 0	±0 12	±1[illegible]	±1 1
		18 8	6 0	1 89	5	10 0
TI/PS (CHF)	11	±1 1	±1 4	±0 [illegible]4	±9	±1 6
MI/AI or AS (CHF)	2	[illegible]1 3	—	3 36	119	14 5

* Ascites free

mately 6 mm of Hg After pulmonary stenosis was established certain of the animals demonstrated a further rise in right ventricular end diastolic followed in 2 weeks by an elevation in left ventricular end diastolic filling pressure The changes in the left heart followed the appearance of ascites In the animals unsuccessfully operated upon (lower curve in figure 1) the end diastolic pressures on the right side remain only moderately elevated and the pressures on the left side remain normal Some of these later animals had transient ascites Simultaneous left and right ventricular pulse tracings for a control dog and for 1 whose congestive heart failure was due to TI/PS are shown in figure 2

The net body weight amount of ascites cardiac index coronary flow and oxygen usage by the myocardium for the 2 control and 2 experimental groups are shown in table 1 Both the animals with inferior vena cava ligation and those with tricuspid insufficiency showed a below normal reduction in cardiac output whereas those with left heart disease showed an increase in cardiac output Coronary flow was not significantly changed from normal in any of the groups and the oxygen usage by the myocardium was likewise within normal limits

The evidence for congestive heart failure in these animals may be seen by examining table 2 in which the atrial and ventricular pressures are reported Both the normal control animals and those with inferior vena cava ligation controls showed essentially normal pressures in both chambers of the heart and no evidence of regurgitation into the right atrium The animals with tricuspid insufficiency and pulmonary stenosis had a moderate right ventricular systolic hypertension (44 mm vs 30 mm for the normal) and markedly elevated end diastolic filling pressures Regurgitation into the right atrium was marked in these animals with the atrial systolic pressure reaching an average of 30 mm of Hg The left ventricle of these animals was also failing indicated by the elevation of end diastolic pressures in that chamber In the 2 animals with left heart disease there was evidence for left ventricular failure in terms of a marked elevated end diastolic filling pressure Some elevation of filling pressure was also noted in the right ventricle

Physicochemical Studies

Results of the physicochemical studies are shown in figures 3 4 5 and 6 and are summarized in table 3 The data in figure 3 show the dependence of sedimentation constant (*upon concentration) for myosin isolated* from the control dogs (normal and ICL) and from those with congestive heart failure The $s^{\circ}_{20,w}$ for the control animals was 6 16 ± 0 13 and for the animals in failure was 6 50 ± 0 01 a difference that is on the border line of significance ($p = < 0\,05 > 0\,01$) The concentration dependence of $s_{20,w}$ is linear The slopes of the sedimentation curves from normal and failing myosin are significantly

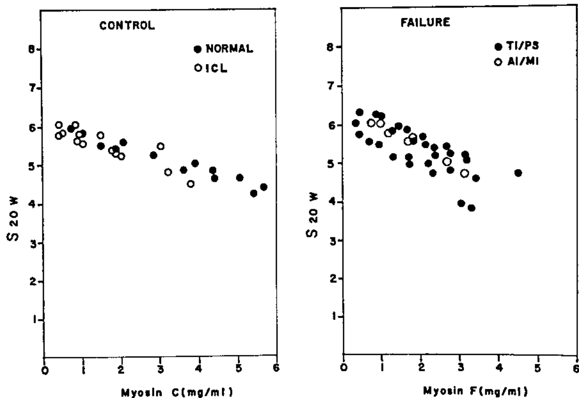


Figure 3

Sedimentation constants of dog heart myosin a a fun tion of concentration The panel on the left shows the values for control animals The open circles are animals with ascites resulting from inferior vena cava ligation The panel on the right shows the values for animals with congestive heart failure The solid circles are from animals with primary right heart failure and the open cir les are from animals with primary left heart failure Conditions 56,100 rpm temperature 4 C 0 6M KCl pH 6 8

different The calculated slope for the normal control group was −3 10 ± 0 16 whereas for the dogs with heart failure it was −6 66 ± 0 84 ($p = < 0\ 01$) The diffusion measurements for normal cardiac myosin and myosin isolated from dogs with heart failure are shown in figure 4 The concentration dependence of $D_{0\ w}$ was more marked with normal cardiac myosin than with myosin from the failing heart On extrapolation to zero protein concentration the value for $D_{0\ w}$ for the normal dogs was found to be 2 46 × 10⁻ cm /sec whereas that for the dogs with heart failure was 0 82 × 10⁻ cm /sec The molecular weight calculated for normal cardiac myosin from sedimentation constant diffusion constant and partial specific volume was 226 000 for myosin from the failing heart it was 680 000

In figure 5 are plotted the turbidities uncorrected for dissymmetry at 90 degrees against protein concentration obtained in the light scattering experiments for myosin from normal and failing hearts The zero intercept of $\frac{H \times c}{\tau}$ is proportional to the reciprocal of the molecular weight After applying correction factors for dissymmetry to the respective intercepts the molecular weight for normal cardiac myosin was found to be 270 000 whereas that for failing cardiac myosin was 760 000

The changes in intrinsic viscosity are presented in figure 6 The shape of the plots

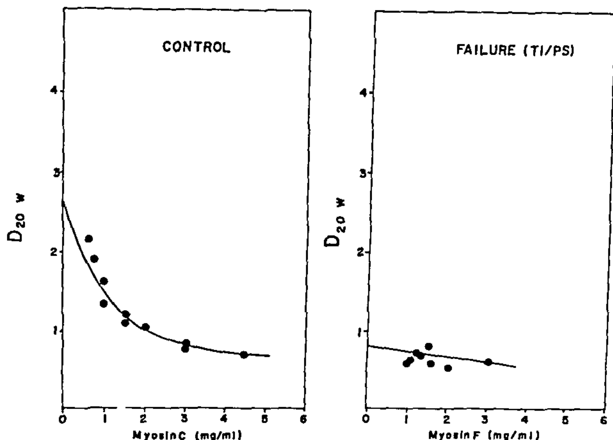


Figure 4

Boundry spreading and free diffusion constants of dog heart myosin as a fraction of concentration The values in the left panel were obtained from normal animals the values in the right panel from animals in congestive heart failure Conditions tempera ture 0 9 C 0 6M KCl pH 6 8

($1/c \ln \eta'/\eta_0$ vs concentration) are markedly different for the 2 proteins Normal cardiac myosin appears to disaggregate in very dilute solution and extrapolates to a limiting value of 50 c g s units On the other hand myosin isolated from the failing heart shows a reciprocal behavior and appears to undergo aggregation in dilute solution to a high limiting viscosity number of 363 c g s units This behavior has also been noted by Davis et al [18]

The ATPase activity of the myosins from normal and failing heart muscle were not significantly different The value for normal myosin was 382 ± 68 microliters P/mg /hr vs 424 ± 112 for the myosin from the failing heart

From analyses carried out thus far it would appear that the amino acid composition of myosin from normal heart failing heart and rabbit skeletal muscle are essentially indistinguishable on the basis of moles/100 000 Gm protein Representative data are shown in table 4 These data strongly suggest that the changes in molecular weight and other properties noted among the myosins represent changes in secondary and tertiary structure

Discussion

The studies demonstrated conclusively that generalized cardiac failure can be induced in the dog by surgical production of tricuspid insufficiency and pulmonic stenosis Elevation of end diastolic filling pressures in the left heart as well as the right heart were noted in most of the dogs that have developed heart failure in our laboratory and all of these ani

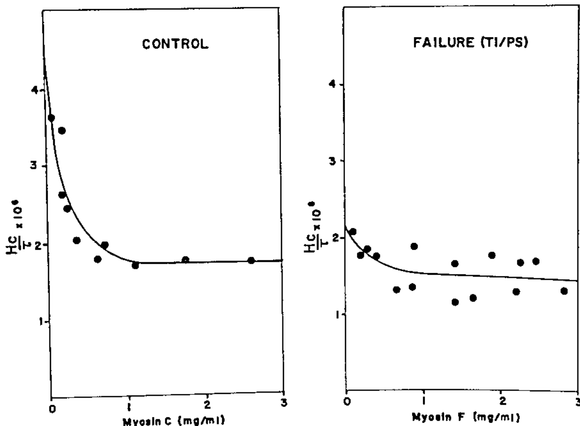


Figure 5

Light scattering measurements on dog heart myosin as a function of concentration The values in the left panel were obtained on preparations from control animals The values in the right panel were obtained on preparations from animals with congestive heart failure Conditions temperature 12 to 15 C 0 6M KCl pH 7 2

mals selected for the study of cardiac myosin from the failing heart This finding supports the studies of Barger Roe and Richardson[19] of heart lung preparations made from hearts obtained from animals whose clinical cardiac failure was due to TI/PS They observed that such hearts failed quickly in vitro in a Starling heart-lung circuit Not only were the hearts unable to maintain normal output when the right atrial venous supply was increased but also they were unable to maintain a normal output when the Starling resistance was increased These authors concluded that On the basis of two experiments it would appear that as in cardiac failure in the human the involvement of one chamber may predominate early in the disease but with the progression of the disease decompensation of both chambers becomes apparent

Our studies are in contrast to those of Davis et al [0] in which the surgical production of tricuspid insufficiency and pulmonic stenosis lead to an elevation of right ventricular but not left ventricular end diastolic pressures These workers concluded that they had isolated right ventricular failure in their preparations We can only conclude that the preparation they obtained was somewhat different from ours They report that right ventricular systolic pressures were rarely increased in their animals whereas these pressures were uniformly increased in ours It is conceivable that the valvular dis

Table 2

Intra ardia Pre sures in Contr l and I rperimental Dogs

C ditl n	No	Right t l p ir Syst	Right t l p ir M n	V nt lc l r pres es (mm Hg) Right Syst	V nt lc l r pres es (mm Hg) Right D t	V nt lc l r pres es (mm Hg) L ft Sy t	V nt lc l r pres es (mm Hg) L ft D t
		C o	3 2	29 4	2 1	165	5 6
Normal	12	±0 C	±0 7	± 8	±0 8	± 4	±1 5
		0	3 1	3? 0	3 4	183	6 3
ICI	5	±0 4	±1 1	±3 7	±0 4	±14	±0 3
		30 1	14 4	44 2	13 6	1?9	11 8
TI/PS (CHF)	11	±3 4	±0 1	±? 1	±1 2	± 7	±1 1
MI/AI or AS (CHF)	2	6	? 0	38 0	? 0	164	14 5

ease was more severe in our animals with extension of the defect in contractility to the opposite side a phenomenon frequently seen in man [1] and expected on the basis of the anatomy of the heart

The molecular weight of normal canine cardiac myosin appears to be about 226 000 from data based on 3 independent methods carried out in our laboratory [6] The valu obtained from light scattering is slightly highly (270 000) but this is typical of a technic that estimates weight average rather than number average molecular weight On the other hand the molecular weight obtained from studies of myosin from the failing heart is 690 000 from D s and $\overline{v}$ and 760 000 from light scattering measurements The ratio of corresponding molecular weights for normal and failing myosin is 1 3 Since the amino acid composition is essentially identical it appears that the myosin from the failing heart is a trimer of that from the normal heart It we designate normal cardiac myosin as myosin C (for cardiac) and myosin from the failing heart as myosin F (for failure) the transformation may be summarized as follows

$$3 \text{ myosin C} \rightarrow \text{myosin F}$$

The stimulus for this transformation seems to be chronic stretch It may be that distortion of the myosin rodlet (thick filaments) in the chronically stretched myofibril may be instrumental in stimulating mild denaturation of normal myosin which in turn results in polymerization Since the ATPase activity of myosin F is not altered the association apparently does not involve the active site of this enzyme Evidence for alteration in the myosin filaments in the failing heart are now being sought in our laboratory by electron microscopy

It seems clear that the change observed represents an acquired molecular disorder that may account for the decrease in contractility of the failing heart The phenomenon of polymerization of myosin has been observed to occur in vitro [3] during denaturation The myosin of rabbit skeletal muscle may furthermore represent a polymer of a monomer of approximately the size of normal cardiac myosin Kielley and Harrington [4] found that guanidine salts will depolymerize rabbit skeletal myosin to a monomer of molecular weight 219 000

Benson[2] studied the effect of heart failure in dogs with tricuspid insufficiency and pulmonic stenosis upon the properties of actomyosin The animals with experimental heart failure possessed a cardiac myosin with reduced ATP sensitivity that is a reduced change in the specific viscosity of actomyosin after addition of ATP These investigators also found that the cardiac myosin peak from experimental dogs seemed to be more prominent in velocity sedimentation studies of actomyosin than in normals They suggested that the combination of actin with myosin was less stable in the experimental dogs as compared with the controls

Davis and his group [5] attempted to verify these findings but were unable to they observed no difference in the ATP sensitivity

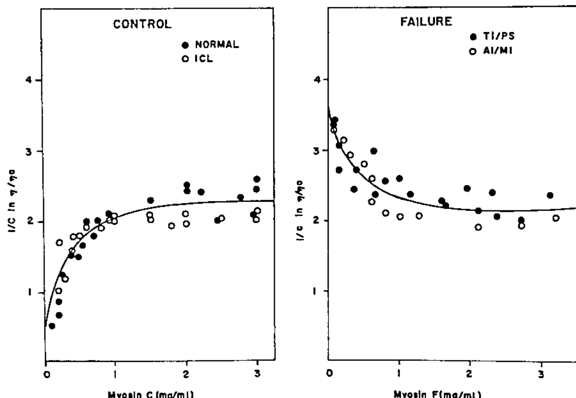


Figure 6

Viscosity measurements on dog heart myosin as a function of concentration. The values in the left panel were obtained on preparations of control animals. The values in the right panel were obtained on preparations from animals with congestive heart failure. Conditions: temperature 1 C, 0.6M KCl, pH 6.8.

of actomyosin from normal heart muscle and heart muscle obtained from dogs with right heart failure resulting from tricuspid insufficiency and pulmonic stenosis. They did observe, however, that certain preparations of actomyosin from the failing right ventricle heart muscle did show a slow component (probably myosin) present in the sedimentation pattern that was not present in controls. In other words, the behavior of actomyosin from the failing heart was not identical with that from normals. Nevertheless, these investigators concluded that these data do not support the concept that the contractile proteins are altered in experimental heart failure.

With regard to the work on actomyosin, it is probable that actomyosin formed during extraction of cardiac muscle by saline phosphate solution is a physiologic artifact. The electron microscopic studies of intact muscle fibers [7] suggest that actin and myosin are segregated in a particulate structure involving the thick and thin filaments in the living cell.[28] They appear to make contact only in a highly oriented manner during the contractile cycle. For this reason the studies of actomyosin, although controversial as noted, probably are not too informative about the state of the contractile proteins in the intact heart.

In the study reported more recently,[18] Davis and his co-workers undertook the characterization of cardiac myosin from normal dogs and from dogs with chronic congestive heart failure caused by tricuspid insufficiency and pulmonic stenosis. Their basic data are in fairly good agreement with ours as reported

Table 3

Properties of Myosin from Normal and Failing Dog Heart

Const nts	C di c Myosin No m l	Falli g
•	6 16	6 53
ds/dc (weight %)	−3 10	−6 66
D ₂₀ •	2 46	0 8°
$\overline{V}$	0 73	0 72
[η] (c g s units)	50 0	363 0
f/fo	2 15	4 56
M (from s D)	°6 000	690 000
M (from light scattering)	70 000	763 000
a/b	°4	80
Length (Å)	690	2 224
Width (Å)	28	28
ATPase (μL P /mg /hr)	382	424

Table 4

Distribution of Selected Amino Acids in Various Myosins (Gm moles/10⁵ Gm)

Ami cid	Dog h t mus l Norm l	Failure	Rabbit k letal mu le K mi s 1954	B iley 1948
Aspartic	83	79	85	67
Serine	39	39	41	43
Glutamic	149	144	155	150
Alanine	71	71	78	73
Valine	36	35	42	·°
Phenylalanine	27	26	27	°6
Lysine	84	78	85	81
Arginine	48	44	41	4°

above They employed a narrower range of physicochemical methods to characterize the myosins from normal and failing heart muscle than have been employed in the present study For example they did not carry out equilibrium sedimentation or light scattering measurements on their preparations Davis and co workers did measure sedimentation coefficients over a reasonable range of concentration and their $s_{w\ 0}$ intercepts are in reasonable agreement with ours We find a higher mean slope with the preparations from the failing heart although the scatter is greater than with the normals It is to be noted however that the sedimentation behavior is a relatively insensitive parameter for distinguishing normal and failing myosin because the sedimentation behavior is relatively unaffected by end to end aggregation

Davis et al admit furthermore the presence of impurities in some of their preparations stating that in a few instances a very small boundary which sedimented faster than the principal myosin component was observed *If this is true other impurities not* distinguishable from the main peak, which could have modified sedimentation behavior at higher concentrations of the preparations from the failing heart could have been present [9]

The measurements of the diffusion constant by Davis et al were not carried out over a sufficient range of concentration to detect the concentration dependence in very dilute solution [6]

The changes in intrinsic viscosity observed and described by us were also observed by Davis and co workers They were particularly notable in observations made by them on myosin from right ventricular tissue from dogs in congestive heart failure An elevated intrinsic viscosity was consistently found In a few preparations a high intercept was noted for the normals although this was not as frequent nor as marked as in the animals with failure Davis and co workers choose to ignore these viscosity findings which are in good agreement with ours and concluded that their viscosity measurements were unreliable 'The possibility must be considered " Davis states 'that viscosity measurements at very low concentrations do not represent the true viscosity of the solution The fact that their viscosity measurements were carried out at room temperature rather than at 1 C makes it likely that denaturation of their normal preparation would occur with a higher frequency, which could account for the occasional higher intercepts noted in control preparations

Without the additional support of measurements of light-scattering and equilibrium sedimentation behavior of these myosins Davis et al drew the conclusion that the molecular weight of cardiac myosin was in the range of 5 × 10⁵ and that there were no

differences between the normal and failing heart We do not believe these conclusions are tenable in view of Davis s own data and particularly in view of the extensive work reported in this communication

Whether or not there are differences in the animal preparations must be considered Davis s animals were sacrificed without the benefit of an open chest and artificial respiration Whether or not the degree of failure is different in the 2 groups of animals as evidenced by the apparently normal end diastolic filling pressures on the right side in the Davis series can be resolved only by further experimentation It is hoped that some reconciliation of these differences will be ultimately effected

With regard to the question of generalized cardiac failure in dogs with TI/PS valvular disease Benson[30] found that in vitro contractility of glycerol extracted muscle strips from both the right and left ventricles of dogs with heart failure resulting from TI and PS was markedly reduced as a depressed ventricular function curve similar to that observed in vivo could be constructed from the data Since glycerol extracted muscle retains little else than the basic contractile system and responsiveness to ATP it seems reasonable to assume that the defective contractility observed by Benson and co workers must be due to altered contractile proteins Kako and Bing[31] observed a similar decrease in contractility of actomyosin bands prepared from failing human heart muscle post mortem when compared with control preparations

It appears from these studies and ours reported herein that cardiac myosin is altered in physicochemical properties in association with congestive heart failure in the dog The extent to which this is etiologic is not at this moment determined Also the extent to which this system is a model for the human with valvular disease is an interesting subject for speculation

Acknowledgment

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Contractile Proteins of Heart Muscle in Man

By Richard J. Bing, M.D., and K. Kako, M.D.

This report deals with the contractile proteins of human muscle in congestive failure and with the role played by the contractile proteins and by biochemical processes in the regulation of the mechanical function of the heart. The contractility of actomyosin bands prepared from heart muscle of patients who had died in congestive failure was diminished as compared to those prepared from normal hearts. This may have been the result of defective protein synthesis. The increase in heart rate was correlated with the activity of phosphorylase a in heart muscle and with changes in carbohydrate intermediates (lactate, glucose 6 phosphate [G 6 P] and glycogen). The heart rates over 300 per minute were associated with a transient increase followed by a decrease in phosphorylase a activity, glycogen diminished while lactate and G 6 P increased. The oxidation-reduction potential in heart muscle became more negative. In the absence of myocardial anoxia the increased rate of stimulation of the heart produced no alterations in either the concentration of carbohydrate intermediates or the phosphorylase a activity. Alterations in function of the heart that come into play upon rapid changes of cardiac activity are the result of the integration of several diverse biochemical cellular reactions. The contractile proteins are but following the lead of the cellular elements concerned with the production of energy.

CONTRACTILE PROTEINS of Heart Muscle in Man is the topic assigned. Strict adherence to this title would limit the field to human heart muscle alone and would make this primarily a technical discussion. Therefore little opportunity would be afforded for dealing with problems of broader physiologic significance. Consequently although the title of this presentation will be adhered to in principle, it will also be used as a starting point to contrast the role played by the contractile elements and by biochemical processes in the regulation of the mechanical function of the heart.

Contractile Proteins and Regulation of Cardiac Function

When one considers the properties of actomyosin in solution, it appears that this protein is most susceptible to the influence of adenosine triphosphate (ATP) and ions. In the presence of ATP at low salt concentrations there is complete dissolution and dissociation.[1] Then with an increase in potassium chloride superprecipitation occurs.[1] As one increases the concentration of potassium chloride further, complete dissolution and dissociation suddenly take place again. Superprecipitated actomyosin forms a gel that can be compressed into threads. Weber called these preparations thread models.[2] He showed that muscle models shorten by 80 per cent of their initial length and that they develop tension. Thus the contraction of the living system and the contraction of these models agree in many points. We do, however, miss evidence of physiologic insight on the part of these models. They contract, they lift weight, and they develop tension, but their response to weight and their speed of contraction are uniform and not adjusted to the demands of the moment.

More than 10 years ago with these considerations in mind we undertook a study of the properties of models prepared from heart muscle both of animals and of man. The question that we tried to answer first was: Do models prepared from heart muscle retain some of the physiologic insight of the intact heart muscle, for example, what is the relationship between the tension devel

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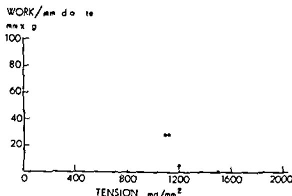


Figure 1

This shows the isotonic contractions of extracted heart muscle after the addition 0 9 per cent ATP The work of the isotonically contracting muscle strip calculated per unit fiber (millimeter length per millimeter diameter) is related to the tension exerted on this preparation The work performance increases with rising tension up to an optimal value and decreases when the tension becomes excessive (From Taeschler and Bing [3])

oped and the work and between the speed of contraction and tension[2] Dr Taeschler using the fiber model preparation of Szent Gyorgyi (the glycerinated heart muscle fiber) found that the work of the extracted heart muscle increases with rising tension up to a maximal value and then decreases as this load is exceeded (fig 1*) [3] In this respect extracted heart muscle reacts similar to fresh heart or skeletal muscle or for that matter to the whole heart

This observation which was interpreted as evidence that the molecular orientation of the contractile proteins of heart muscle during stretch determines the work performance prompted us to extend our studies to the contractile elements of the human heart [4] If our conclusions were correct then similar technics should enable us to accumulate evidence of an altered state of contractile proteins in heart muscle of patients who had died in congestive failure From our metabolic studies we had already reached the tentative conclusion that in heart failure excluding such types as beriberi or thyrotoxic heart disease the disturbance may be in the contractile proteins [5] If one could prepare actomyosin models from failing human hearts one might supplement the studies of Olson [6] Davis [7] and Benson,[8] who had attacked this problem by means of biophysical and physical chemical technics

The success of this work depended on 2 unknowns (1) are available actomyosin models suitable for this work and (2), since the studies were to be based on the contractility of actomyosin bands obtained from hearts after the death of the patient it would have to be shown first that characteristic properties of actomyosin do not change for a brief period after death Dettli explored both these problems in answer to the first question he found that actomyosin threads produced by the compression of surface spread fibers of the proteins possess certain definitive disadvantages [9] In a thick thread such as the one prepared by Hayashi most of the molecules within the thread are not exposed to immediate contact with ATP but must be reached by unequal diffusion of ATP from the bath [10] In addition the thickness of the thread varies a great deal making it difficult to obtain uniform results Dettli overcame these difficulties by compressing actomyosin spread on the surface of a solution into bands not into threads [9] This afforded a more constant diffusion of ATP into the preparations and made them a useful tool in comparative experimental studie (fig 2) Dettli also devised an apparatus for the measurement and recording of after loaded isotonic contractions in this system refined by Kako it is not necessary to handle the sticky actomyosin band directly instead the band can be loaded by moving the weighing spring of a torsion balance by the desired number of milligrams (fig 3*) [4 9] Since the band contracts on the addition of ATP the arm of the torsion balance follows the con

*Figure 1 reproduced from Taeschler and Bing Circulation Research 1 129 1953 By permission of the American Heart Association Inc

*Figures 3 and 4 reproduced from Kako and Bing J Clin Invest 37 463 1958 By permission of the American Society for Clinical Investigation

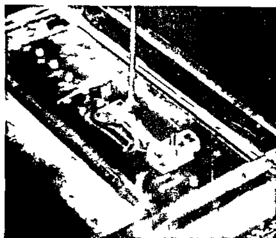


Figure 2
This illustrates an actomyosin band in the contraction chamber. One end of the band is anchored firmly while the other is attached to the lever of a torsion balance.

traction. This initiates an electronic servo feedback mechanism which moves the trough in a direction opposite to that of the contraction. Thus the arm of the torsion balance is always kept in the equilibrium position and a counter force is produced that equalizes the tension of the thread.[4] The movements of the trough are recorded and without appreciable friction it is possible to register shortening of the band at a magnification of 23 diameters.

The second problem, possible changes in the contractility of actomyosin after death, was studied by Dettli for the dog's heart and by Kako in actomyosin bands prepared from human hearts.[4, 9] Kako found that the contractility of actomyosin bands remained undiminished for at least 6 hours after the death of the patient.[4]

The way now appeared open for a comparison of the contractility of actomyosin bands prepared from the left ventricles of normal and of failing human hearts. The actomyosin bands prepared from heart muscle of patients who died in congestive failure were found to possess diminished contractility (fig. 4). It is not within the scope of this paper to dwell on the reasons for this dimin-

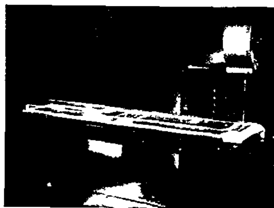


Figure 3
The Langmuir trough in which the actomyosin solution is compressed into band. The contraction chamber is at the right. The torsion balance and its elongated arm are also shown. As the protein band contracts, the arm of the torsion balance moves with the contraction, the trough moves in an opposite direction. Thus the counterforce produced equalizes the tension on the bands. (From Kako and Bing.[4])

ished contractility on a molecular level, but the actomyosin bands have an advantage over actomyosin solutions in that studies on the essential property of the contractile proteins, their contractility, can be carried out. The results on actomyosin bands prepared from human hearts are in agreement with those of Benson who, using glycerinated heart muscle strips from failing myocardium of dogs, found that these fibers did less work than fiber bundles from normal hearts under equivalent conditions of length, temperature, pH and ATP concentration.[10]

We have asked ourselves repeatedly about what factors could cause a change in contractile elements of heart muscle leading to diminished contractility. We favored the hypothesis of stretch as playing an important role, but this has never been conclusively demonstrated. A change in orientation of actomyosin is also unlikely since Olson observed changes in myosin molecules of failing heart muscle.[6]

Recently a very interesting observation on failing hearts has been published by Meerson and Zayats.[11] Their report is of particular

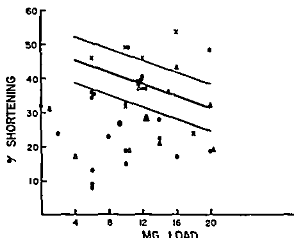


Figure 4

The contractility of actomyosin bands prepared from failing human hearts and the effect of digoxin and of calcium and digoxin combined The regression line and standard deviations obtained from normal data are represented Many of the points obtained from failing hearts are below the standard deviation of the normal Digoxin does not influence the percentage shortening (the group mean is still below the deviation of normal data) The combination of digoxin and calcium results in marked improvement of contractility indicated by the fact that the group mean is now on the normal regression line □=Control b=—0 879 p<0 01 •=failure control b=—0 019 p>0 9 Δ=digoxin b=0.95 p>0 1 ×=digoxin + Ca b=0 404 p>0 5 (From Kako and Bing [4])

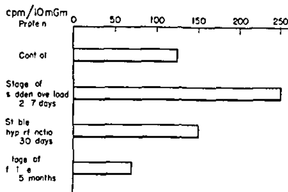


Figure 5

Data from Meerson and Zayats[11] are graphically illustrated in this figure The rate of incorporation of S^{35} methionine into myocardial protein increases immediately after the production of aortic insufficiency and then decreases during the state of stable hyperfunction it reaches its lowest level during myocardial failure

interest since it touches on the previous discussion while leading into the regulation of cardiac function by biochemical processes These authors measured the rate of protein synthesis in hearts of rabbits with experimentally produced aortic stenosis [11] Protein synthesis was determined by the rate of uptake of S^{35} labeled methionine into the heart muscle The changes occurring in protein synthesis during the development of failure are illustrated in figure 5 Immediately after the production of aortic stenosis a period called the "state of sudden overload the heart dilates and its weight increases The rate of protein synthesis doubles and microscopic changes in heart muscle are noticeable Muscle glycogen and creatine phosphate diminish while lactic acid concentration in heart muscle rises During the second stage that of stable hyperfunction the heart weight first increases then remains constant and the rate of protein synthesis returns to normal There is hypertrophy of the muscle fibers The myocardial concentration of phosphocreatine and glycogen is normal but the lactic acid concentration remains elevated During the third stage that of cardiac decompensation the heart weight remains stable but there is dilatation and protein synthesis decreases (fig 5) Lactic acid concentration in heart muscle increases creatine phosphate diminishes while glycogen concentration remains unchanged Accordingly cardiac hypertrophy produces an increased myocardial mass and an increase in sarcosomes Myocardial anoxia is present as illustrated by the increase in lactic acid The authors conclude that the disturbance in protein synthesis in the myocardium is an important factor in the development of myocardial failure and that the loss of kinetic energy of cardiac contraction is connected with a disturbance of the normal process of protein synthesis in heart muscle The cause for diminished protein synthesis may be prolonged anoxia with reduced ATP synthesis or a deficiency of deoxyribonucleic acid (DNA) the latter brought about by a relative increase in the size of the cytoplasm as compared to nuclear mass [11]

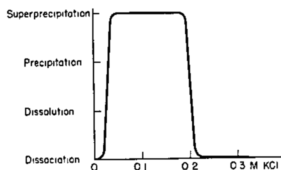


Figure 6

At very low potassium chloride concentrations and in the presence of ATP actomyosin is dissociated As the potassium chloride concentration is increased superprecipitation occurs As the ionic strength is further increased dissociation and dissolution again take place (After Szent Gyorgyi[1])

The Role Played by Biochemical Processes in the Regulation of Cardiac Function

The challenging concept of Meerson and Zayats relating possible alterations in contractile elements to protein synthesis leads us into the second portion of the discussion a consideration of biochemical events concerned with the functional regulation of the heart muscle It is not difficult to predict the influence of these biochemical factors They are likely to be responsible for a greater speed of contraction and should play a predominant role in the adaptation of the heart muscle to rapid changes in internal environment Actomyosin bands gels superprecipitations have with few exceptions no ways of grading their responses except in an all or none fashion Superprecipitation is an example As mentioned before in the presence of ATP a slight increase in KCl causes an intense precipitation provided one starts at low potassium chloride concentrations If 2 consecutive test tubes differ by no more than 0 02 molar potassium chloride dissolution is found in 1 superprecipitation in the other (fig 6) In all likelihood it is the presence of the cell membrane that is responsible for a smooth regulation of the intracellular ionic concentration In addition to the presence of a membrane or membranes the organelles of

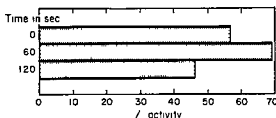


Figure 7

The effect of ventricular fibrillation on active phosphorylase activity of the left ventricle is shown Active phosphorylase activity first increases then diminishes as ventricular fibrillation proceeds Mean value of 4 experiments

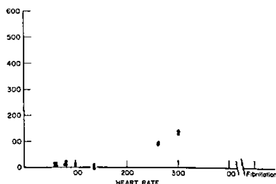


Figure 8

The rise in heart rate results in an increase in lactate concentration (μM/100 Gm muscle) of left ventricular muscle The highest concentrations are found in ventricular fibrillation

energy production the mitochondria regulate the speed of contraction in vivo Their ubiquitous presence makes ATP accessible to every portion of the fibril while in artificial models the contraction depends on the penetration of ATP from the bath solution

Therefore although actomyosin bands may present true models of the contractile elements they are stereotyped and devoid of the ability to respond quickly and to adapt themselves independently to alterations in environment

The relationship between enzymatic activity at the cellular level of organization and the functional activity of the heart muscle may be investigated by integrating changes in either the rhythm or the force of con

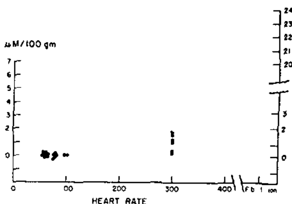


Figure 9

Increase in the heart rate is accompanied by a rise in the glucose 6 phosphate concentration of left ventricular muscle The highest values are found in ventricular fibrillation

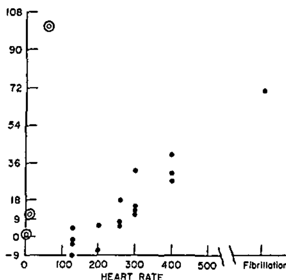


Figure 10

Increase in the heart rate leads to a diminution in myocardial glycogen concentration during first ~ minutes (mg/100 Gm muscle) As compared to skeletal muscle glycolysis occurs at much faster rates of contraction ◎=Skeletal muscle (Cori) ●=heart muscle

traction of the heart with well defined biochemical reactions In 1943 the Coris discovered the enzyme phosphorylase which catalyzes the reaction glycogen + inorganic phosphate = glucose I phosphate [1] The enzyme exists in an active and in an inactive form enzymes in muscle can convert the active into the inactive form or can catalyze the reverse reaction [12] Phosphorylase appears therefore as an important enzyme in determining the rate of glycogenolysis in skeletal muscle Cori summarizing the effect of stimulation on phosphorylase a content of skeletal muscle stated that increasing the speed as well as the total number of contractions causes a progressively greater increase in the amount of active phosphorylase however during tetanic contractions the ratio of phosphorylase a to total phosphorylase diminished [12]

An increased rate of stimulation of skeletal muscle also results in major changes in the carbohydrate intermediaries of the Embden Meyerhof cycle Thus, in anaerobic muscle glycogen disappears while lactate and hexosephosphate accumulates apparently phosphofructokinase becomes the rate limiting step during anaerobic contraction [1] If the response of the heart muscle and of skeletal muscle is similar then the increased heart rate should result in glycogenolysis with accumulation of glucose 6 phosphate (G 6 P) and lactic acid it should also lead to an increase in the relative concentration of phosphorylase a to total phosphorylase This supposition is correct but applies only to the anoxic heart muscle

Figure 7 illustrates that ventricular tachycardia with beats over 300 per minute and ventricular fibrillation first cause an increase and then a diminution in phosphorylase a activity [13] Similar results are obtained in atrial muscle during atrial fibrillation [13] The increase followed by the fall in active phosphorylase can be explained by assuming that during the first seconds of tachycardia, the heart muscle is still alkaline favoring the enzyme that synthesizes phosphorylase a [1]

The increased heart rate is also accompanied by definite changes in carbohydrate intermediates [13] As in skeletal muscle the increased rate of contraction leads to an increase in lactate and G 6 P concentration and to a decline in glycogen (figs 8 9 and 10) This suggests that, as in skeletal muscle the enzyme phosphofructokinase is the rate limiting enzyme under these conditions Undoubtedly anoxia as initiated by a decline in coronary blood flow is present under these

circumstances this is confirmed by a more negative oxidation reduction potential as calculated from the ratio lactate to pyruvate [13]

The shift to an anaerobic metabolism and to glycogenolysis and to the transient increase in phosphorylase activity are the results of quick adaptive reactions in energy production the actomyosin band or thread lacks this ability it is the fly wheel of the steam engine without the steam generator

In the absence of myocardial anoxia an increased rate of stimulation of the heart produces no alterations in either the concentration of carbohydrate intermediates or in the phosphorylase a activity (table 1) [13] These results were obtained in hearts in situ in which coronary arteries were perfused from a donor animal Apparently the increased rate of stimulation in the absence of anoxia fails to evoke the metabolic pattern described as typical for muscular contraction An increase in heart rate alone is not sufficient to stimulate the activated enzyme [13]

Likewise the increased force of contraction for example as initiated by angiotensin is without effect on phosphorylase a activity [13] This enzyme therefore appears to be activated only under the influence of anoxia or catecholamines which has been shown by Meyer and Moran and by Kukovetz and others [14 15]

The conclusions that may be drawn from this discussion are of a specific and of a general nature In the first place whatever the underlying physical chemical reasons may be actomyosin prepared from failing human heart possesses diminished contractility This adds some weight to the argument that the fundamental defect in heart failure lies in the organs of energy utilization It is intriguing to consider a causal relationship between defective protein synthesis and diminished contractility

In general alterations in function of the heart that come into play upon rapid changes in demand are the result of the integration of several diverse biochemical reactions in the

Table 1

Percent Changes in Phosphorylase A as a Result of Atrial Fibrillation and of Ventricular Fibrillation after Perfusion of the Coronary Arteries

A ti phosph ryl % f total			A t phosph ryla % f total			
Tim	[illegible]	[illegible] †	Tim	[illegible]	[illegible]	[illegible]
Exp			Exp			
13		1	17	?		0
14	46	44	18	31	31	9
15	8	31	19	37	37	38
16	4	46	0	40	43	38
Mean	34 5	35 5	Mean	3? 5	33 3	31 3
± S E	± 5	± 5 9	± S E	± 4 0	± 4 5	± 4 3

Left atrial appendage
†Right atrial appendage

cell which are regulated by the cell membrane and the substructures of the cell concerned with energy production The contractile proteins are but following the lead and command of those cellular elements concerned with energy production and those endowed with the regulation of ionic transfer into and out of the cell

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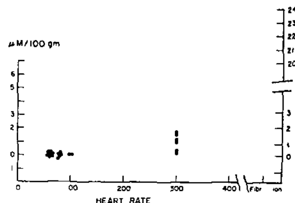


Figure 9

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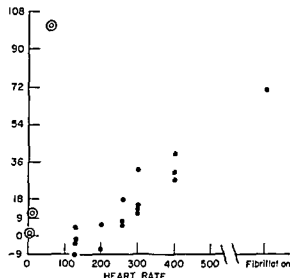


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Discussion

Chairman Eichna Dr Konigsberg does ATP cause contraction of these early cells either in the mononucleated phase or in the 3 or 4 day stage when cross striations appear?

Dr Konigsberg The multinuclear ribbons begin to contract spontaneously at about the seventh day of culture By this time cross striation is apparent These contractions occur however without the addition of exogenous ATP

We have added ATP to the glycerol extracted cultures and these respond as one would expect of glycerol extracted models upon the addition of ATP they contract You start out with a culture that is about the size of a quarter and get a dime in change

Dr Mommaerts Dr Konigsberg it struck me that some of the effects of nitrogen mustard persist over several days yet they leave a substance in water that persists only for about 15 minutes Is the implication that the cells are influenced during this short time and that this influence persists?

Dr Konigsberg Yes exactly We prepare the mustard solutions immediately before use and expose the cells for an hour although we realize that this may be longer than necessary The first 15 minutes as you point out would probably be sufficient We then replace the nitrogen mustard solution with complete growth medium What we measure subsequently is the damage that was done during those first 15 minutes

Dr DeHaan Dr Konigsberg have you ever redissociated the syncytia before striations are formed from the glass and will they form single cell suspensions again?

Dr Konigsberg The difficulty we have experienced is that upon the addition of trypsin to differentiated cultures we get clumps of interwoven multinucleated ribbons in addition to free cells These clumps have thus far resisted dispersion If we remove the clumps by filtering the cell suspension through bolting silk and plate the free cells we again get the formation of multinuclear cells—although they are sparse Several repetitions of this procedure eventually yield muscle free fibroblast cultures

Dr DeHaan Well then do you get the impression that these syncytia once they form do not disaggregate into single cells?

Dr Konigsberg No I don t think so We are not convinced that trypsin disaggregates the multinuclear ribbons although Rinaldini has suggested that it does (*Exper Cell Res* 16 477 1959) Godman and Murray (*Proc Soc Exper Biol & Med* 84 668, 1953) applied colchicine and noted that the multinucleated cells broke up into smaller fragments that later re associated DeRenyi and Hogue (*Arch exper Zellforsch* 16 167 1934) observed contracting multinuclear cells that gave off mononucleated buds during this period of contraction We think that a similar breakdown into mononucleated elements occurs during the early stages of muscle regeneration The multinucleated muscle cell may represent a biologic metastable state that is *completely reversible* under the appropriate conditions

Dr Fishman Dr Konigsberg would you be willing to say if you think that these observations also apply to cardiac muscle? Have these experiments been done on cardiac muscle?

Dr Konigsberg My former collaborator Dr William Cooper is studying cardiac muscle I think that he will probably get very similar results One question of interest is whether there is a stage in developing cardiac muscle corresponding to that of developing skeletal muscle in which rows of contiguous nuclei form I would suspect that this arrangement wouldn t occur in cardiac muscle because it isn t a true syncytium The intercalated disc as I understand it is an actual physical separation between what were originally individual cells

Dr Richard L Klein (Jackson Mississippi) Do you see *intercalated* discs in embryonic cultures?

Dr Konigsberg We have worked only with skeletal muscle I don t know *

Dr Klein (Jackson, Mississippi) Dr DeHaan in view of your proposal of 2 distinct cell types in cardiogenesis you are undoubtedly familiar with the work of Fange Persson and Tesleff Would you care to comment on the fact that myocardial cells when cultured in tissue culture after trypanization apparently will show a typical pacemaker type of potential up to 8 days?

Dr DeHaan Yes I am familiar with their work[39]† and it is very interesting I m glad you asked this question because it gives me an opportunity to expand on the apparent lability of pacemaker function in immature myocardium As you ve suggested clusters of embryonic ventricular cells when isolated in tissue culture pulsate spontaneously and exhibit pacemaker prepotentials Recordings taken from such clusters from 4 day hearts always show *diastolic depolarization*[38] *In con*trast Meda and Ferroni[61] have demonstrated that similar recordings from intact embryonic ventricle in situ do not show pacemaker prepotentials Finally Fange Persson and Thesleff[39] demonstrated that cell clusters from 8 day ventricle sometimes but not always exhibit pacemaker prepotentials Thus it would seem that in early stages of development cells capable of functioning as pacemakers are widespread throughout the heart but that in the normal course of development their spontaneous activity is suppressed or masked When subjected to culture conditions they once again exhibit prepotentials Later in development that is at 8 days it would appear that some cells have now been fixed as ventricular myocardial units and have thereby lost their ability to exhibit prepotentials even under culture conditions This is the only reasonable explanation that I can think of at the moment

Dr Mommaerts May I return to the high glycogen again?—because that is still very mysterious You indicate, of course that this high glycogen makes one think of glycolytic metabolism But to explain it, i e, the high content we need a second fact that is some form of periodic use because glycogen is a storage food and we cannot understand its function unless there are periods in which it is used and other periods in which it is resynthesized For example by analogy, we find *starch deposited in the roots of trees during* the fat season and then used during the lean spring when leaves have to grow Later on starch is replaced Unless we find similar periodicity in the use of glycogen in the heart we will be unable to understand why it is there

Dr DeHaan This is a most interesting topic which I have not worked on myself However to add fuel to your argument Schiebler[1] states that the glycogen in the conduction system is more resistant to a period of anoxia than is glycogen in the myocardium that is even under prolonged periods of anoxia glycogen disappears more slowly from the conduction fibers than from the ventricular muscle On the other hand, the enzymo*logic information that I referred to earlier* suggests that conduction tissue has a relatively low rate of oxidative metabolism These are the observations as they now appear in the literature I m afraid I can t go beyond them

Dr Rhodin I should like to hear a comment about the ability of the conducting system to contract Do you know of any studies in which the conductive cells have been raised in pure culture?

Dr DeHaan No To my knowledge the culture of conductive cells has not been done I am in fact at present getting organized to do tissue culture and have considered attempting just this project There are statements in the literature to the effect that the bundle and bundle branches of some *species* when dissected out are relatively noncontractile

* Since this symposium was held a note has appeared describing the development of cardiac muscle cells from disaggregated cell suspensions (*Science* 132 1839 1960)

† References in Dr DeHaan s discussion identified by superior number will be found in *References* at the end of his paper

even when stimulated Perhaps Professor Weidmann would comment on this

Dr Weidmann Excised Purkinje fibers of ungulates (sheep calf horse) usually do not contract even when looked at through the microscope although they may generate and will always propagate action potentials However by increasing the calcium concentration in the bathing fluid 2 or 3 fold an action potential will invariably be associated with a visible contraction This indicates that even the most differentiated conducting tissues have retained the potential for exciting a mechanical response under suitable conditions

Dr Arthur H Briggs Dr Olson from your studies of cardiac myosin in congestive heart failure do you believe that your animals are in a stage of irreversible failure and whether any new insights could be gained by giving digitalis or perhaps by releasing some of the pulmonic stenosis?

Dr Olson In general we have not been able fully to reverse this failure either by sodium restriction or by the use of diuretics or of digitalis We have done a few preliminary studies on the effect of digitalis upon myosin isolated from the hearts of both normal animals and animals in failure We have shown that digitalis is bound to myosin (*Fed Proc* 18 221 1959) Although the effects on the protein are variable there is one consistent feature that is the digitalis tends to make myosin more unstable This may have some implications for our hypothesis If in fact trimers are not so well utilized in the contractile mechanism as monomers something that would produce even disorganized fragments of the polymerized myosin might conceivably be beneficial to the subject with heart failure

Another point is that with heart failure resulting from tricuspid insufficiency and pulmonic stenosis we do not have a good model to test the effects of inotropic agents upon sodium retention Stimulation of the right ventricle to greater contractility with digitalis usually further elevates the right atrial pressure and may lead to further sodium retention We are going to restudy this problem in animals with primary left heart failure which should give a better answer to your question and hopefully a clearer picture of the effects of digitalis upon myosin

Dr Ellis S Benson (Minneapolis Minn) One has a natural desire to defend one s work to the hilt but there is also the need to be objective Dr Olson has referred to our work on actomyosin and on glycerol extracted trabecular muscle preparations from the chronically failing heart In both of these preparations one must realize that we are dealing with a rather unstable poorly characterized preparation subject to much inherent variability

Dr Olson and Dr Bing present an interesting case for the existence of changes in the myocardial contractile proteins in chronic congestive heart failure I think that on the basis of our experience in this field I would urge an attitude of caution in drawing conclusions pending further investigation

Dr Bing Dr Huxley based on your work on the sliding in and out of actin and myosin fibers what do you believe is happening during prolonged stretch? After all the failing heart is stretched for a long time What happens during chronic stretch?

Dr Huxley I think that the degree of overlap of the filaments would obviously be less when a muscle is working in prolonged stretch In the particular model that I am supporting I see no reason to suggest that the prolonged stretch would be detrimental to the muscle Beyond that I wouldn t say anything

Does anyone know the amount of myosin per gram of muscle in these failing hearts?

Dr Olson We haven t studied this in a really quantitative way because our objective was to obtain a purified sample for characterization From the size of the precipitates at various stages of purification however I would say that the difference between the normal and the failing heart with regard to myosin is not marked

Is there any evidence Dr Huxley that in chronic stretch the A band changes at all?

Dr Huxley As I say I haven t looked at muscle in chronic stretch

Dr Olson I ask this question because it

seems possible to me that some degree of depolymerization inside the A band may be a feature of a normal heart This depolymerization could be a feature of the organization of the protein in the thick filament, terminating in the double strands of myosin which protrude and show as 'feet If there were stresses that might distort the A band filament which I think we have to accept now as a sort of organelle this distortion in turn might cause internal changes in the state of organization of the protein itself and result in polymerization This is the kind of idea we have been speculating about

Dr Huxley Is there any effect of chronic stretch on any other muscle than heart muscle?

Dr Olson We are in the process of studying that now in the rabbit and the dog We are trying to extend the femur in such a way that we can get a chronically stretched leg muscle for study It would be interesting if we could generalize the hypothesis of polymerization with chronic stretch to skeletal muscles

Dr Mommaerts Before coming to my question I must express doubt that chronic stretch of skeletal muscle occurs under biologic circumstances even if you put people on the rack you are only stretching their ligaments aren t you?

Dr Olson *This is the reason why we have* to rely on the surgeon again The gross difference between cardiac and skeletal muscle in the intact organism is that the skeletal muscle has an origin an insertion and a fairly fixed range of motion.

Dr Mommaerts To start with a small technicality the molecular weight of skeletal myosin in our preliminary paper was 380 000 but in the final paper when we applied a correction for concentration dependence of light scattering the weight increased to 420 000 plus With the exception of one recent paper by Harrington and Kielley (*Biochim et biophys acta* 41 401 1960), where it has gone up higher this value of 420 000 to 450 000 is now being found by just about everybody both as a molecular weight and also as a binding equivalent for 1 molecule of ATP This *discrepancy will have to be explained*

I can only say that the data that Dr Olson has presented look completely convincing There has of course been controversy on this point but what we have seen here leaves no place for any other conclusion but that there is a different myosin in the failing and in the normal heart

Now as to his concept as well as that of Dr Bing—that this difference in myosins characterizes heart failure They are saying something very different from what I said yesterday when I stated that heart failure is a disease of the force velocity relation But this relation must have a mechanism and, in chronic cases this relation could very well be along the lines suggested by Dr Olson In other words what sounded so different yesterday is only a difference of emphasis and not in any fashion a contradiction of the concept that we have just heard

Dr Olson I should like to add 1 point about the findings of Harrington and Kielley which give a molecular weight for skeletal myosin of about 620 000 Again this could be an artifact of preparation I don t think it is an artifact of physical chemistry With guanidine salts Harrington and Kielley have been able to depolymerize their skeletal myosin into a particle of 216 000 molecular weight *which in particle size matches very closely* the cardiac myosin that we have isolated from the heart although the physical constants indicate that it is more unfolded It could be that their particle is the fundamental particle in the myosin series of the order of 220 000 in molecular weight In vivo this particle could give rise to molecules with different degrees of polymerization Some such polymers could be formed under pathologic conditions We know that even more extensive polymerization can occur in vitro as a result of denaturation

Question from Audience Dr Olson in your slide summarizing the differences between myosin obtained from normal and failing hearts you included a statement for ATPase activity you stated that this was not

a significant difference although it appeared to be significant from here Would you comment on this?

Dr Olson The question was what about the ATPase values for the normal and failing heart? Actually the range of activity that has been obtained in the study of the normal and failing heart is such that a statistical analysis of the 2 groups of values shows no significant difference It would thus appear that the active site for ATPase is not altered per unit weight which one might assume is reasonable if the polymerization does not involve linkages around the active site

Perhaps a more important fact is that skeletal myosin has a much higher ATPase activity That has been found consistently and is also true of the heavy myosin fragments after tryptic digestion

Another point of difference is that we really cannot find heavy myosin fragments in our products of tryptic digestion of cardiac myosin

Dr John Gergely (Boston Massachusetts) Dr Olson has made an excellent case for both the low molecular weight of normal heart myosin and the high molecular weight of myosin obtained from failing hearts However I find it rather difficult to set aside the results of Davis and his colleagues (*J Clin Invest* 39 1463 1960) at the National Institutes of Health and also those of our laboratory (Gergely J and Kohler H *Fed Proc* 16 185 1957) which consistently indicate a molecular weight of normal heart myosin of around 450 000 I should add that since we used the light scattering technic which was preceded by extensive centrifugation at high dilution I cannot quite see how our preparations could have been contaminated with actomyosin

However setting this aside I should like to make a constructive suggestion putting two and two together that is putting together what Dr Mommaerts said in one of his comments about their extremely ingenious method of measuring ATP binding and the ideas of various degrees of polymerization that Dr Olson has brought up

If Dr Olson is right then one should find that in heart myosin the combining weight of myosin with ATP is about 200,000 and this would not be affected by the presence of any small actomyosin impurities or aggregation We have made some measurements on the binding of pyrophosphate (which can serve as model substance) to heart myosin and we found that 1 mole of pyrophosphate combined with about 500 000 Gm of protein Perhaps Dr Mommaerts would do these experiments and by his technic we could have a quick and decisive answer Naturally the fact that the combining weight for ATP of skeletal myosin is about 400 000 to 500 000 would seem to rule out a simple dimer picture for the skeletal protein

Dr Olson We have thought about these studies Perhaps Dr Mommaerts and I can collaborate in applying the firefly technic to the study of this binding constant for normal cardiac myosin

The problem of a difference in molecular weights obtained for cardiac myosin in different laboratories may be physiologic rather than physicochemical We have observed for example that in normal animals allowed to suffer anoxia during the period of sacrifice polymerization of myosin occurs and higher particle weights are obtained in normal animals If one allows time to elapse before the heart is chilled in ice water at 1 degree one finds changes that affect particle size in other words there seems to be a very labile system for post mortem polymerization This should be explored further

Question from Audience Could Dr Olson comment further about the time element with reference to the human studies where studies of heart myosin are done 2 or 4 hours post mortem?

Dr Olson We all appreciate that it is exceedingly difficult to obtain human cardiac muscle shortly after death It is possible as Dr Bing has shown that the actomyosin fibril no matter how it is composed in vitro will contract if isolated any time up to 6 hours after death What the size of the myosin monomer is under these conditions of course,

is unknown I would doubt that it remains unchanged for 6 hours post mortem Nevertheless we are going to try to study human cardiac myosin under various conditions Possibly we can control the viability of the preparation by plotting a curve of mitochondrial phosphorylation or some other index as evidence for the intactness of the muscle and extrapolate to zero time

Dr Furchgott I should like to comment again on this matter of whether there is a deficiency in the conservation of energy in the high energy phosphate pool In my paper yesterday, you may remember I commented on the work of Drs Feinstein and Schwartz on the hearts of guinea pigs in chronic failure due to aortic stenosis They found respectively a fall in high energy phosphates especially creatine phosphate and about a 30 to 40 per cent uncoupling of oxidative phosphorylation I was cautious about interpreting their results and did not conclude that the fall in efficiency of oxidative phosphorylation and levels of high energy phosphate produced the failure These defects may have come about subsequent to a failure as a result of various other changes But I should like to stress that if such defects are associated with failure they would certainly aggravate the situation making for an even worse condition of the myocardium than would have existed in their absence

I was interested to hear about the results of the Russian workers which Dr Bing reported Apparently in the later stage of failure they too found a fall in creatine phosphate Is that right?

Dr Bing You are correct During the first stage (which they call the accident stage) when the heart dilates rapidly Drs Meerson and Zayats observed a fall in creatine phosphate but no change in ATP A second diminution in creatine phosphate and also a decrease in ATP occurred during the final stage that of gradual decompensation

Dr Furchgott It may be equivalent to what Feinstein finds in the markedly hypertrophied hearts of guinea pigs in severe congestive failure

Dr Bing I agree with Dr Furchgott that there are different types of mechanisms which lead to heart disease Lack of high energy phosphate may be one of them Other situations that may start the development of heart failure may be truly metabolic such as occurs in beriberi heart disease

Dr Olson I should just like to emphasize Dr Furchgott that at least in our experimental preparation there is no evidence in the state of advanced heart failure for any decrease in CP storage The mitochondria in these animals seem normal in vitro This is not to say that one can't get a mixed lesion, although I think even in the guinea pig there has been a report by Plaeut and Gertler (*Ann New York Acad Sc* 72 515 1958) that mitochondria from guinea pigs in failure from aortic stenosis do phosphorylate normally so I think that it is possible that more studies of chronic animals may reveal a component of anoxia resulting from coronary ischemia This is just a suggestion but I think it is very clear that one can have cardiac failure without a change in oxidative phosphorylation

Dr Furchgott I agree that you could have it All I m saying is that there may be examples of failure—even clinical failure—in which this is an additional aggravating factor I m not saying that it is the initial cause of the failure

Dr Olson Yes I have just 1 other comment about Dr Bing's talk if I may with regard to the Russian work on incorporation of S^{35} methionine into total protein An alteration in the contractile proteins and a change in S^{35} methionine uptake are not necessarily mutually exclusive phenomena It may well be that this abnormal protein does not turn over so fast in fact we know that myofibrillar proteins turn over very slowly indeed

Dreyfus Kruh and Schapira (*Biochem J* 75 574 1960) studied the uptake of C^{14} glycine into the skeletal myofibril in the rat and found the turnover to be remarkably slow Further the slope of the isotope decay curve suggested that the myofibril is like a red cell undergoing little decay but only dissolution Further changes could occur in failure

DISCUSSION

Dr Huxley I was wondering if anyone had tried to make heavy myosin from this low molecular weight and if so what does its molecular weight come out to be?

Dr Olson We have tried it and failed

Dr Hoffman I wonder if Dr Bing would elaborate a little bit on the study reported from the Russians on the decreased incorporation of sulfate into protein? Is it possible that *this change results in any way from the altered* nutritional status of an animal that has had aortic stenosis for many months and presumably may have been near death at the time of the study?

Then also on his own data I wonder if he would elaborate a little on the extent to which changes in phosphorylase activity are related either to anoxia or to the release of catecholamines? If it is primarily anoxia how much anoxia independent of catecholamine action do you need to produce these changes?

Dr Bing Meerson and Zayats have excluded the possibility of the nutritional status of the animal as contributing to defective protein synthesis in heart muscle This they have done by showing that the changes in protein synthesis in heart muscle are not paralleled by synthesis of plasma protein As for Dr Hoffman s second question there appears little doubt that the changes in phosphorylase activity are related to anoxia What role catecholamines play in this connection is not clear since we have not performed any determinations of catecholamines under these circumstances

Dr Paul F Cranefield (New York N Y) If you do the same experiment that was mentioned that of cross circulation the heart which is put into fibrillation shows no deteriorating changes in its electric activity and the single-cell potential except for the changes resulting from the fact that the rate is very

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V Electrophysiology

Chairman Chandler McC Brooks, Ph D

Introduction

By Chandler McC Brooks Ph D

IN THE closing session of this symposium we are to consider a function that actually precedes the mechanical contractile responses we have heard so much about The heart beat originates in a pacemaker where spontaneous depolarization occurs Conduction of excitation involves a transmission of this depolarization over a specialized conducting system and then ultimately to the basic contractile fibers of the myocardium

Dr Weidmann in starting the afternoon s meeting will discuss membrane excitation in cardiac tissue Dr Hoffman will then describe the studies which he and Dr Paul Cranefield have carried out recently on the conduction process in the specialized conducting tissue in their paper we shall be turning back to a matter that has been discussed at some length by previous speakers I think that by

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the end of this symposium we shall see clearly that 3 or 4 major problems have been emphasized One of them will be the problem of transmission in the conducting system and another will be the meaning of the intercalated disc

Our third subject of the afternoon will bring us again to the matter of contraction this topic will also involve a discussion of membrane phenomena

The final presentation of the day will be devoted to a completely new subject Dr Hajdu will be concerned with materials that affect the contractile process In brief we will begin this afternoon with a new approach to the problem of cardiac function then as the afternoon progresses we will reconsider topics that you heard discussion of yesterday and this morning Our session will begin with Professor Silvio Weidmann of Berne who will describe the processes involved in Membrane Excitation

The Experimental Basis of Concepts

Einstein s theory of relativity the most magnificent achievement of modern physics was suggested by closest adherence to experimental facts this is its strength We may admire the grandeur of its structure of thought and the depth of its ideas but this alone would never have secured for it that firm position in physics which it enjoys today This position was secured because it is able to explain experimental facts to foretell events it was the later confirmation of these events which made this theory great —H Reichenbach *From Copernicus to Einstein* New York Philosophical Library 194 pp 51 52

Membrane Excitation in Cardiac Muscle

By SILVIO WEIDMANN, M.D.

The contributions made during the past 10 years are reviewed. Intracellular recording has made it possible to state absolute values for the cardiac resting potential (90 mv., inside negative to outside) and the overshoot during activity (30 mv., inside positive to outside). The surface membrane of a resting fiber is considered to be predominately permeable to K ions. During activity Na conductance increases and K conductance decreases. The latter process is thought to be essential for explaining the high membrane resistance that is measured during the long-lasting plateau found with cardiac muscle. A hypothesis is presented that would account for the termination of the plateau and the beginning of repolarization.

IT IS THE PLAN of the present survey to begin with a description of the electrical events during cardiac activity, to continue with their interpretation in terms of the movements of ions, and to close by treating a more special problem: the possible reasons for the long-lasting action potential that is typical for cardiac muscle.

Intracellular Recording

It was in 1949 that Ling and Gerard[1] working in Chicago described a new tool that stimulated electrophysiologic research in almost all its branches: the capillary microelectrode. Two people who had been taught the technic by Ling were to become responsible for extending the method from skeletal to cardiac muscle: J. W. Woodbury, then working at Salt Lake City, and A. L. Hodgkin of Cambridge, England. I can well remember the day of July 16th, 1949. Having learned the microelectrode technic at Cambridge and having been rather unsuccessful in prodding around in different tissues of the frog, I became a heart physiologist by 2-fold chance: from 5 to 6 p.m. Dr. Feldberg had demonstrated a Starling preparation to the medical students and allowed me to cut out the dog's heart, and my wife agreed that I need not be home for supper at 6:30.

Now what is the advantage of the new technic? The suction electrode introduced and widely used by Schutz[4] had revealed the time course of the cardiac action potential with a fair amount of accuracy. Only by the use of the Ling-Gerard electrode, however, was it possible to record the full amount of the potential difference existing between the inside and the outside of a cardiac fiber.

Figure 1 illustrates the potential changes that can be observed when a microelectrode is introduced into a rhythmically driven preparation (sheep ventricle). The zero line or reference potential is first recorded between two *extracellular* electrodes (see drawing upper right). One of the electrodes then is moved into the preparation. Touching and penetrating the endocardial layer causes minor potential changes (first arrow). The penetration of a muscle fiber by the electrode tip (second arrow) is signaled by the occurrence of large potential changes synchronous with the contractions. In diastole the potential difference is constant (resting potential here 85 mv.). On pulling back the electrode the potential drops to the reference level. When viewed on a faster time base (fig. 1, lower records) the transmembrane action potential shows an extremely rapid upstroke (1 msec.) followed by a plateau and a moderately rapid downstroke (repolarization).

The upstroke would coincide with the QRS complex and the downstroke with the T wave of a surface ECG.

The Distribution of Ions

In heart muscle, as in other living cells, the ionic composition of the inside differs marked

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Supported by grant 1977 from the Swiss National Science Foundation.

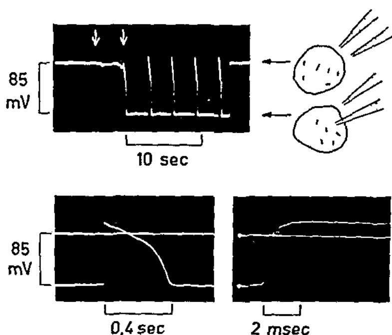


Figure 1
Upper record *Potential changes as observed during the introduction of a Ling Gerard electrode into a myocardial fiber The electrode positions corresponding to the 'zero and to the transmembrane potentials are seen on the upper right* Lower record *From the same preparation at higher sweep speeds to bring out a single action potential (left) or its initial phase (right)*

is from that of the extracellular space There are different views on how the inside composition is maintained in spite of the established fact that the membrane is permeable to all the ionic species that have to be considered (fig 2) One of the simplest hypotheses is this Na ions leave the cell by some pump process of an unknown nature Na outflux takes place against an electrical gradient and a concentration gradient for thermodynamic reasons it requires metabolic energy K and Cl ions may be looked upon as being passively distributed In the case of K ions the force from the concentration gradient (outward) would be balanced by the force from the electrical potential gradient (inward for a positive ion) It may be stated that the ratio of the K concentrations 30 1 (inside outside) and possibly also that of the Cl concentrations 1 30 (?) are in good agreement with the measured potential difference of about 90 mv (for references see Weidmann[5] and Hoffman and Cranefield[6])

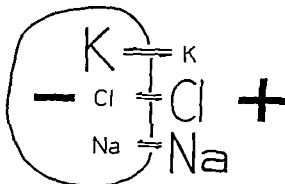


Figure 2
Distribution of ions between the inside and the outside of a muscle fiber Exchange at rest for various ions is indicated with arrows of different length

The Movement of Ions

If the inner surface of a cell membrane is to become more positive as during the upstroke of an action potential the positive charge must be shifted from outside inward During repolarization on the other hand the positive charge must leave the cell Identification of the ionic species that carry the charge has been accomplished on a quantitative scale for the giant nerve fibers of the squid mainly by the Cambridge group [7] Experiments on cardiac tissue then revealed many similarities (for references see Hoffman

and Cranefield[6]) Thus it is generally agreed that the movements indicated by figure 3 are responsible for the cardiac action potential inward movement of Na ions for depolarization outward movement of K ions for repolarization These shifts may be passive that is they may be brought about by a succession of permeability changes first a transitory increase of Na permeability then a slight increase of K permeability

The state of ionic concentration differences represents stored energy and makes it possible for strong ionic currents to flow during the action potential At the end of activity the inside of the fibers will have gained a minute quantity of Na ions and lost a similar quantity of K ions If ionic order is to be maintained over a longer period Na ions must be ejected and K ions accumulated during the time between the two action potentials

Evidence for an Increase in Na Permeability During the Action Potential

Since the work of Overton[8] it has been known that Na ions are necessary for cardiac excitation Figure 4* shows an experiment of the type that led to the conclusions expressed in the preceding section Replacement of 80 per cent of the normal NaCl by choline chloride—choline being a nonpenetrating ion—has the following effects (a) the resting potential remains unaltered suggesting that the resting membrane is but sparingly permeable to Na ions (b) the amplitude of the action potential and its duration decrease suggesting that normally there is an inward Na current during activity Furthermore the upstroke velocity of the action potential can be shown to be roughly proportional to the extracellular Na concentration[9, 10] suggesting that during the initial phase of activity Na ions are the main carriers of charge

The Plateau of the Cardiac Action Potential

In a nerve fiber the action potential is ended in less than a millisecond in the mammalian ventricle activity lasts for a few tenths of a second During the plateau phase there is but little change in the membrane potential as a function of time a state which means that inward current is almost equal to outward current A further analysis will be facilitated if more is known about the membrane resistance Figure 5 shows (left) an experiment performed on a sweet water alga kept in Upsala tap water (method of resistance measurements[11]) During the plateau of its long lasting action potential the resistance is low and a detailed analysis[1] of data from the alga *Chara* has revealed that during that phase a strong potassium inward current is balanced by a strong chloride outward current With mammalian ventricular fibers the

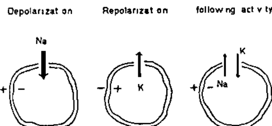


Figure 3

The shifts of ions leading to the changes of the membrane potential during electrical activity Restitution of ionic gradients after activity

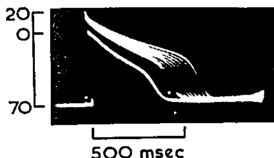


Figure 4

Evidence in favor of low Na permeability at rest and high Na permeability during activity Successive action potentials of the same fiber of sheep ventricle The vascular bed of the preparation was perfused first with normal Tyrode solution then with a test solution containing 1/5 of the normal sodium The camera was opened during the first 30 seconds after the admission of the Na poor solution then again between 60 and 90 seconds (From Délèze[9])

Figure 4 reproduced from Délèze Circulation Research 7 461 1959 By permission of the American Heart Association Inc

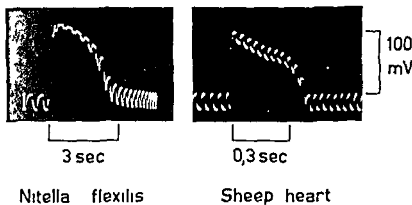


Figure 5
Measurement of membrane resistance during activity Square pulses of hyperpolarizing current were applied to the preparation The resulting voltage change is a measure of resistance Left *With* Nitella flexilis, *a sweet water alga low resistance on plateau* Right *With sheep myocardium, high resistance on plateau*[17]

resistance during the plateau is relatively high (fig 5) This is an important finding which indicates that the increase of the Na permeability causing the upstroke of the action potential is at least partially reversed while the permeability to other ions remains constant or even decreases

Data of a more quantitative nature have been obtained for Purkinje fibers of the sheep heart (fig 6) * The action potential of Purkinje fibers regularly shows an initial spike and thus a comparatively low plateau, as a rule there is some potential drop during diastole which is typical for all membranes

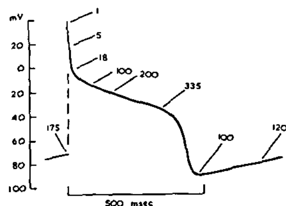


Figure 6
Membrane resistance during activity of a sheep Purkinje fiber for comparison with figure 9 Relative resistance values are indicated for different phases of the action potential (From Weidmann[18]*)*

Figures 6 and 10 reproduced from Weidmann Ann New York Acad S 65 663 1957[18] By permission of the New York Academy of Sciences

with pacemaker properties The figures attached to the tracing indicate that the resistance is even higher during activity than at rest

The behavior of the nerve membrane was adequately described by Hodgkin and Huxley[7] with the aid of a set of empirical equations Briefly it was assumed that depolarization causes a transitory increase of Na conductance (G_{Na}) and a long lasting but somewhat delayed increase of K conductance (G_K) The increase of G_{Na} would be responsible for the inward current causing depolarization the decrease of G_{Na} with a simultaneous increase of G_K would provide the outward current causing repolarization

An attempt to produce a Purkinje fiber action potential by applying the Hodgkin Huxley equations was recently made by Noble[14] (fig 7) * In choosing appropriate parameters the high membrane resistance of Purkinje fibers during the plateau could be simulated only if G_K was allowed to *fall* as a consequence of depolarization The conductance changes computed by Noble s machine are seen in figure 8 * G_{Na} rises as a consequence of depolarization after the initial spike it settles down at about 8 times its resting value G_K by contrast falls as the driving force for K^+ outflux increases

Figure 7 (left) reproduced from Draper and Weidmann J Physiol 115 74 1951 [6] and figures 7 (right) and 8 from Noble Nature 188 495 1960 [14] By permission of the Journal of Physiology and of Nature

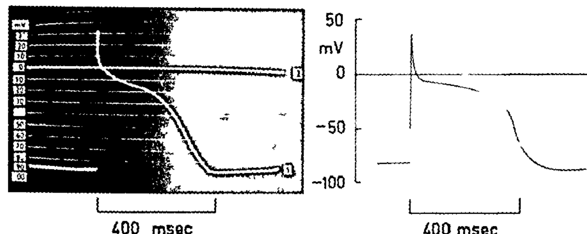


Figure 7

Left *Recorded action p t ntial of a lo) Pu kinje fiber (From Draper and Weid mann*[16]*)* Right *Similar a tion potential computed with the aid of the Hodgkin Huxley equations (From Noble*[24]*)*

Convincing experimental evidence is avail able in the case of the nerve membrane to show that G_K rises as a consequence of de polarization[15] It is important then to pro vide experimental evidence for the suggested drop of G_K in Purkinje fibers With this intention membrane resistance was measured over a large range of membrane potentials To minimize the contribution of Na ions the experiments were performed in choline chlo ride (Hutter and Noble [16] Carmeliet[17]) to eliminate even chloride ions as carriers of charge a solution of choline acetylglycinate was used[17] Under such experimental condi tions the membrane current practically has to be carried by K ions

Figure 9 reveals that the membrane re sistance corresponding to a membrane poten tial of −40 mv (plateau level) is indeed 3 to 4 times higher than that corresponding to −90 mv (resting level) This is taken to suggest that the assumption of a low G_K dur ing the plateau is well justified

The Changes Responsible for Repolarization

Applying long pulses of depolarizing cur rent to a Purkinje fiber in a Na free solution Hutter and Noble[16] found a slow decrease of membrane resistance that was complete at th end of a few tenths of a second This change

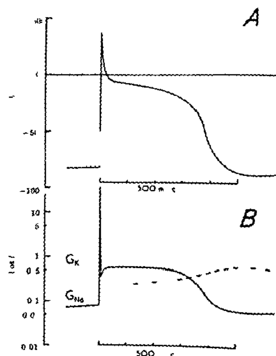


Figure 8

A *Compute l action potential same as in figure The integration was started b; displacing the m mbrane potential to −50 mv* B *Time course of membra ie con luctance plotted on a logarithmic scale* G_K *denotes potass um con lu tance* G_N *sodium con luctance The potassium an l sodium equil ium pote t al were −100 and + 40 mv respectively (From Noble*[24]*)*

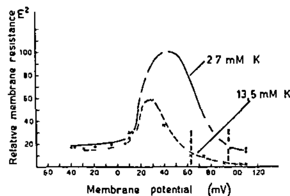


Figure 9

Membrane resistance as recorded from a sheep Purkinje fiber in a sodium free solution. Note high resistance (=low membrane conductance) in the region of the normal plateau. Effect of K rich solution on membrane resistance. Courtesy of Dr. E. Carmeliet, Louvain (unpublished).

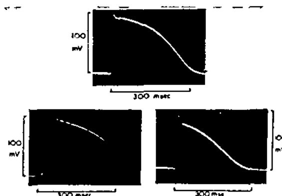


Figure 11

Effect of chloride substitution on the duration of the action potential. Sheep right ventricle. Upper record: *Normal Tyrode solution.* Lower left: *Chloride ions substituted by acetylglycinate.* Lower right: *Chloride ions substituted by nitrate. Courtesy of Dr. E. Carmeliet, Louvain (unpublished).*

might indeed be responsible for repolarization if it is attributed to a rise of G_K thus resulting in a stronger outward current of K ions.

The finding of a strong outward current of tracer potassium (^{42}K) during the phase of membrane repolarization[18] would seem to be in line with the electrical data.

Finally, do we know of any possible reason for which G_K might increase as a function of time when the membrane is held at a constant potential in the region of the plateau? In this connection let me recall that a rapid rise of the extracellular K concentration induces a premature repolarization (fig. 10). One of the suggested mechanisms for this observation was recently tested by Carmeliet.[19] He was able to demonstrate that an increase in the extracellular K concentration causes a drop in membrane resistance that may be interpreted as a rise of G_K (fig. 9). The effect is most pronounced near −40 mv, i.e., in the region of the plateau of the action potential of a Purkinje fiber.

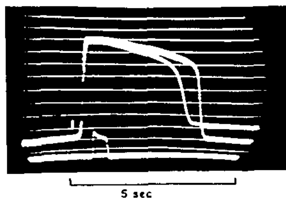


Figure 10

Shortening of the action potential of a turtle ventricle owing to a rapid rise of the K concentration in the perfusate. Potassium-rich solution was made to flow into the coronary artery for half a second, as indicated by the lowest trace. With a delay of 1½ seconds this initiated an early but incomplete repolarization. Voltage calibration lines from 10 to 10 mv. (From Weidmann.[13])

Furthermore Carmeliet[19] measured the influx and outflux of radio K using extracellular solutions of different K concentrations. He could indeed establish that the rate of influx as well as that of outflux became larger when the outside K concentration was increased, again suggesting a rise of G_K.

It seems not unlikely therefore that the plateau is brought to an end by the following mechanism: (a) outflux of K ions during the plateau, (b) accumulation of K ions in a narrow space around the fibers, (c) increase of G_K as a consequence of the rising extracellular K concentration, (d) increase in the rate of K outflux. This is a regenerative process and might well be responsible for terminating the cardiac action potential.

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Physiology of Atrioventricular Transmission

By Brian F. Hoffman, M.D.

This paper describes records of the transmembrane action potential of fibers from different parts of the specialized conducting system and electrograms recorded directly from these fibers in situ. On the basis of these records it is possible to describe certain physiologic mechanisms for conduction delay, block and supernormal conduction. In general impaired conduction is associated with a reduced level of membrane potential. This may be caused by incomplete repolarization or partial depolarization. In the normal conducting system local differences in action potential duration and local pacemaker activity most frequently are the cause of a low membrane potential. In disease states, on the other hand, many other factors may be operative. At the atrial margin of the atrioventricular (A-V) node local anatomic and electrophysiologic properties of the fibers normally cause a very low conduction velocity. The safety factors for conduction here appear to be quite low and delayed transmission or block often does not result from refractoriness or partial depolarization of nodal fibers. Supernormal conduction, at least in Purkinje fibers, seems to result from the high level of membrane potential reached at the end of repolarization. Whether other factors are responsible for supernormality within the A-V node remains to be seen.

DURING the normal cardiac cycle, electrical activity originates in some part of the sinoatrial node and spreads to the atrium and thence to the atrioventricular node. After some delay, activity then spreads through the bundle of His, the bundle branches and the peripheral Purkinje fibers and finally reaches the musculature of the ventricles. In the normal heart activity that is initiated in some part of the ventricles can spread in the reverse direction along this same path. However, under a wide variety of abnormal conditions either sequence of events may not take place. Atrial activity may be delayed excessively at the atrioventricular node or may fail to excite the bundle of His. Activity that does traverse the atrioventricular node may be delayed or blocked in its passage through the specialized conducting system, and this delay or block may be localized to one or another anatomic subdivision. Also unidirectional conduction delay or block may be observed. Finally, most and perhaps all parts of the specialized conduction system may at times develop intrinsic rhythmicity and such ectopic pacemakers may compete with varying degrees of success with the normal sinus pacemaker.

A large number of comprehensive studies of normal and abnormal atrioventricular transmission has been carried out by careful analysis of the electrocardiogram. These studies have provided a fairly detailed picture of the physiology of the various parts of the atrioventricular (A-V) conducting system; however, the evidence in most instances has been somewhat indirect. This is so because electrical activity of the A-V node, the bundle of His, and the Purkinje fibers is not recorded directly in the conventional electrocardiogram. However, 2 methods can be used to demonstrate this electrical activity: through an intracellular microelectrode one can record the change in transmembrane potential associated with activity of a fiber from any part of the heart, and by means of small electrodes placed directly over various parts of the specialized conducting system one can record the local electrogram of the underlying structures.

We have employed these 2 technics to study certain aspects of the electrophysiology of fibers in the A-V node, the bundle of His, and the Purkinje system and to obtain some

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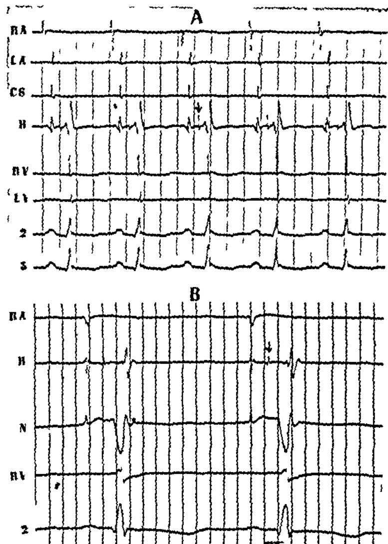


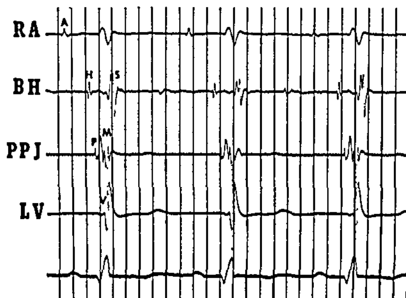
Figure 1

A Bipolar electrograms recorded through chronically implanted electrodes from dog heart and standard electrocardiograms RA right atrium LA, left atrium CS coronary sinus H bundle of His RV right ventricle LV left ventricle 2 and 3 leads II and III Arrow indicate electrogram deflection caused by activity in the bundle of His B Records similar to those in A obtained from a different animal RA bipolar electrogram from the right atrium H bipolar electrogram from the bundle of His N unipolar electrogram from the region of the atrioventricular node RV bipolar electrogram from the right ventricle 2 lead II electrocardiogram For all bipolar electrograms low frequency components (i e under 40 c p s) and components above 200 c p s are strongly attenuated The unipolar electrogram from the node and the electrocardiograms are recorded with the frequency response usual for electrocardiography Time lines at intervals of 40 msec

what more direct information on the physiologic mechanisms responsible for certain disturbances of A V transmission Experiments using microelectrodes were carried out on isolated preparations of rabbit or canine hearts The methods have been described elsewhere in detail [1] Electrograms were recorded directly from various parts of the specialized conducting system of canine hearts in situ using small electrodes that had been attached to the endocardium during total cardiopulmonary bypass [3] The experiments were acute in some instances in others records were obtained from healthy animals in which electrodes had been implanted previously [4]

Activation of the Specialized Conducting System
The Sequence of Activation

The exact sequence of activation of the specialized conducting system in canine hearts has been determined by several investigators from bipolar electrograms recorded by means of electrodes closely placed over the bundle of His the right and left bundle branches and the peripheral Purkinje fibers and from unipolar electrograms recorded by way of electrodes in close proximity to the A V node [5-11] In figures 1 and 2 are several such tracings recorded simultaneously with a standard limb lead electrocardiogram A fairly accurate estimate of the instant during the



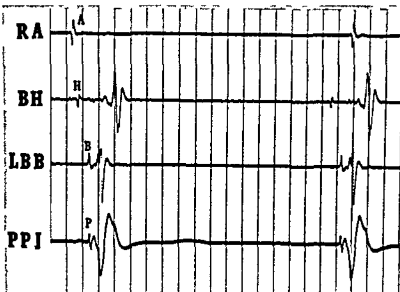


Figure 2

Top *Bipolar electrograms recorded through chronically implanted electrodes from right atrium (RA) bundle of His (BH) right Purkinje fiber–papillary muscle junction (PPJ) left ventricle (LV) and a standard lead II electrocardiogram Deflections are labeled as follows A atrium H bundle of His S septal muscle P peripheral Purkinje fiber M papillary muscle Low frequency components are filtered from the second third and fourth traces Time lines at intervals of 40 msec*
Bottom *Bipolar electrograms recorded during an acute experiment through electrodes located on the right atrium over the bundle of His the left bundle branch (LBB) and the peripheral Purkinje fiber papillary muscle junction Deflections are labeled as in A Time lines at intervals of 40 msec Low frequency components are filtered from all traces*

cardiac cycle when atrial activity reaches the A V node can be obtained by noting the time at which atrial depolarization is recorded through leads located either over the bundle of His or below the ostium of the coronary sinus (fig 1*A*) From such records it is apparent that the A V note is excited early during the P wave of the electrocardiogram The time required for activity to traverse the node can best be determined by recording the onset of propagated activity in the upper end of the bundle of His This approach is necessary because the electrical activity of the A V nodal fibers is not easily demonstrated in surface electrograms [6, 11] Unipolar records from the node show a slow predominantly positive deflection of low voltage (fig 1*B*) however it is likely that in many instances this deflection derives in part from currents associated with repolarization of nearby atrial muscle

Electrodes located over the bundle of His on the other hand clearly signal the arrival of propagated depolarization in this structure The electrogram deflection resulting from activity in the upper part of the common bundle

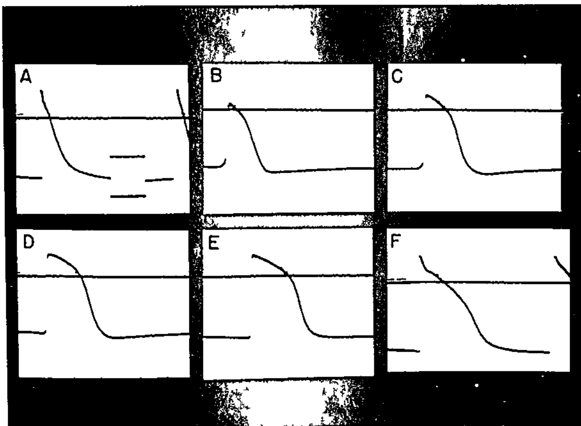


Figure 3

Transmembrane action potentials recorded from single fibers of atrium (A), upper node (B), mid and lower node (C, D, E) and upper of His bundle (F). Upper trace represents a line of zero potential and hou tim calibration (dots) in intervals of 10 and 50 msec. Voltage calibration in A from above down shows in mv —50 and —100. Overshoot in (A) is larger than that commonly recorded. (From Hoffman et al.[12])

usually designated by the letter H, appears during the first half of the P-R interval. Identification of the complexes resulting from activity in different parts of the specialized conducting system often is facilitated if low frequency components of the tracing are filtered. This device allows the use of high amplification to increase the relative magnitude of the rapid complexes that result from activity in the bundle of His or the Purkinje fibers. It has been employed for the records shown in many of the illustrations; in each instance the filter settings of the preamplifier are noted in the legend. The time required for A-V nodal transmission in dogs, determined from records like those in figure 1, ranges from 40 to 50 msec. These findings have been the same both in acute experiments and in animals with chronically implanted electrodes.[4] The electrogram recorded from the bundle of His varies somewhat in configuration and timing depending on the location of the electrodes;[6] however, during normal A-V transmission it is apparent that excitation of the various fibers in the common bundle is quite synchronous.

Records obtained through electrodes located over the right or left bundle branches, the free running Purkinje fibers in the false tendons of the left ventricle, and the peripheral Purkinje fibers at their junction with the papillary muscles of the left or right ventricle can

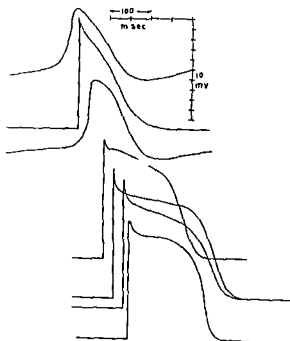


Figure 4

Drawings of transmembrane action potentials recorded from the following sites from above down sinoatrial node atrium atrioventricular node (atrial margin) bundle of His left bundle branch Purkinje fiber in a false tendon terminal Purkinje fiber and ventricular muscle fiber Note the sequence of activation at the various sites as well as the differences in the amplitude configuration and duration of the action potentials (From Hoffman and Cranefield[2])

be employed to time the onset of activity in these structures (fig 2) At each location the rapid deflection resulting from activity in the Purkinje fibers can be identified since the recording electrodes are located progressively closer to the ventricular terminals of the specialized conducting system the interval between this rapid deflection and the slower activity caused by depolarization of ventricular muscle decreases The earliest activity recorded from the right or left bundle branches appears shortly after the midpoint of the P R interval that obtained from electrodes located at the Purkinje fiber–papillary muscle junction often is synchronous with the beginning of the initial deflection of the standard electrocardiogram Electrodes located at appropriate sites on the endocardial surfaces of the ventricles record the local arrival of depolarization in the subendocardial Purkinje fiber network as small rapid deflections that precede local ventricular activity by an interval of a few milliseconds

Conduction Velocity

When the time in the cardiac cycle of electrograms recorded from each part of the specialized conducting system is considered in relation to the distance between recording sites it is apparent that conduction velocity varies considerably during the spread of the impulse from the atria to the ventricles Extremely slow propagation through the A V node has been postulated for many years recent studies of perfused dog hearts[11] and isolated preparations of dog and rabbit heart [1] have shown that, at the atrial margin of the node the conduction velocity apparently falls to the extremely low value of 0 05 M /sec or less During normal A V transmission the major delay in propagation is localized to the atrial margin of this structure Within the node conduction velocity increases progressively and in the bundle of His attains a value of 1 0 to 1 5 M /sec [13] It is likely that the rapidity of spread of the impulse increases progressively toward the periphery of the common bundle Measurements of conduction velocity in the free running Purkinje fibers in the right and left ventricles of canine hearts obtained during cardiopulmonary bypass give values for conduction velocity ranging from 3 to 4 M /sec In the fine terminal ramifications of the Purkinje system conduction velocity decreases and in ventricular muscle velocity is approximately 1 M /sec During retrograde activation which results from premature ventricular activity late in the cycle or from an idioventricular pacemaker firing at a low frequency there is no demonstrable change from normal in the conduction velocity in the Purkinje fibers and in the bundle of His Retrograde transmission from the A V node to the atrium is slower than that recorded during normal activity [11] and again the delay appears to be localized to the atrial margin of the node During such retrograde activation of the specialized con

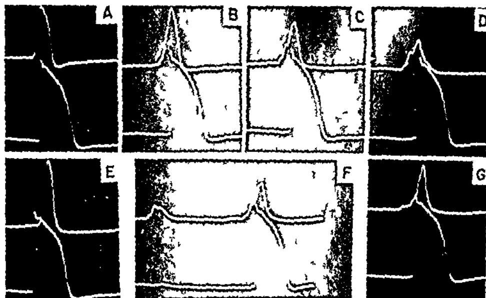


Figure 5

Transmembrane action potential recorded from single fibers of the atrial margin of the atrioventricular node (upper trace) and bundle of His (lower trace) in an isolated preparation of rabbit heart A Control B C effect of acetylcholine added to perfusion fluid Note progressive increase in slurring and notching of upstroke of nodal potential (B D) normal nodal action potential caused by retrograde activation (E) and fragmentation of nodal responses in F and G (From Cranefield Hoffman, and Paes de Carvalho [19])

duction system local electrograms reveal 2 changes The complex recorded from the bundle of His may show some slight asynchrony of activation of various fibers or fiber groups Also the complexes recorded from the region of the A V node show a deflection that is not observed during normal transmission[8] and that may represent slow or delayed activation of atrial fibers at the atrionodal junction

Transmembrane Action Potentials of Different Fibers

Probably the most useful record of electrical activity of cardiac fibers is the record of transmembrane potential obtained through an intracellular microelectrode Transmembrane action potentials have been recorded from single fibers in isolated preparations taken from all parts of the specialized conducting system[7] Unfortunately it has not yet been possible to obtain such records from the heart in situ Also although the peripheral Purkinje system of the adult canine heart has been studied extensively many of the records from the bundle of His and A V node have been obtained from the hearts of rabbits or puppies This resort to small animals is an outcome of the large mass of these structures in the adult dog heart which prevents adequate perfusion of the isolated tissues Nevertheless it is possible to present a reasonably complete picture of the electrical activity of the single fibers present in each of the major subdivisions of the conducting system

The A V Node

Transmembrane action potentials recorded from fibers located in different parts of the A V node are compared to a record of an atrial transmembrane action potential in figure 3 * Several differences are apparent The resting potential of the nodal fibers is lower than that of the atrium the rate of rise of

* Figure 3 reproduced from Hoffman et al Circulation Research 7 11 1959[14] By permission of the American Heart Association Inc

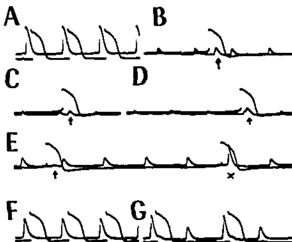


Figure 6

Transmembrane action potentials recorded from single fibers of the atrioventricular node (upper trace) and bundle of His (lower trace) of an isolated preparation of the rabbit heart A Control B D after addition of acetylcholine to the perfusion fluid E G during washout of acetylcholine Note the small depolarization of the nodal fiber resulting from atrial activity and also from activity in the bundle of His (arrows) These subthreshold depolarizations summate (D) *and elicit a nodal action potential if of sufficient amplitude (as at x in* E)

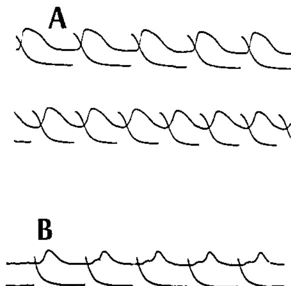


Figure 7

A Transmembrane action potentials recorded from atrioventricular node (upper trace) and atrium (lower trace) of puppy heart showing changes in nodal action potential caused by high rate B *Similar records recorded from a different preparation In this experiment the nodal action potential is recorded somewhat closer to the lower node and the perfusion fluid contains a low concentration of acetylcholine The first action potential is the last beat at a slow rate the subsequent potentials are caused by a rapid driving rate Note the distinct step on the upstroke of the nodal action potential*

the nodal action potential is much less and the overshoot is reduced in amplitude At the atrial margin of the node records of transmembrane action potential often reveal one or more steps or notches on the upstroke [1, 14, 15] The recorded electrical activity differs in different parts of the node [1] the characteristics mentioned are most prominent at the atrial margin and become less pronounced in records obtained from fibers located closer to the bundle of His The duration of the action potential is greater in the lower node than at the atrial margin of this structure and some slow diastolic depolarization is present in all records Studies of conduction velocity within the node[1, 16] have shown that extreme slowing is present only in the fibers at the atrial margin i e in those fibers whose action potentials show the lowest rate of rise and lowest amplitude Both of these properties of the nodal action potential—reduced amplitude and low rate of rise—would decrease conduction velocity It is not known whether the extremely slow propagation of activity in this part of the node results mainly from the characteristics of the action potential or in part also from the anatomic and passive electrical properties of the fibers A V nodal fibers in canine hearts are 6 microns or less in diameter[17] and in some areas have branches or extensions that are still smaller [18] In small fibers other factors being the same conduction velocity is reduced because the resistance to flow of current along the axis of the fiber is high Accurate measurements of the membrane resistance and capacity and the threshold potential of fibers at the atrial margin of the node have not been made and thus it is impossible to evaluate the extent to which the unusual action potential and conduction velocity depend upon these properties How

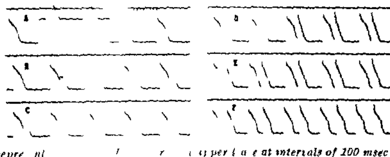


Figure 8

Transmembrane action potentials recorded from an isolated canine Purkinje fiber showing the change in configuration and amplitude of responses to depolarizing pulses applied at various times during and after repolarization Upward voltage step in A repre[illegible] ... per [illegible] at intervals of 100 msec

ever even if one assumes that they ar[illegible] the same as in other cardiac fibers the [illegible] factor for transmission in this part of th[illegible] undoubtedly is reduced

The Bundle of His and the Purkinje Fibers

The action potential recorded from a [illegible] fiber in the bundle of His of the rabbit he[illegible] contrasts quite markedly with the nodal ac[illegible]tion potential (figs 3 and 4*) The resting potential is higher the upstroke of the action potential is rapid, and the amplitude [illegible] at[illegible]er The duration of the action potential is increased and slow diastolic depolarization is minimal under ordinary conditions Th[illegible] rapid depolarization and good amplitude of the action potential in combination with the greater diameter of the fibers undoubtedly are responsible for the increase in conduction velocity as activity spreads into this structure Action potentials recorded from Purkinje fibers in the bundle branches and peripheral branches of the conducting system show other changes There is a further increase in rate of rise and a small increase in the amplitude of the action potential in the bundle branches and false tendons and then a progressive decrease in the terminal Purkinje fiber network The duration of the action potential also increases with increasing distance from the common bundle and then decreas[illegible]s as the junction of the Purkinje fiber with ventricular muscle is approached (fig 4) Diastolic depolarization is progressively less marked in record obtained from more peripheral fibers Comparative data on fiber diameter and frequen[illegible]t branching at different locations in the [illegible] [illegible] system are not available It is likely, [illegible] that both factors (large diameter [illegible] infrequent branching) contribute to the [illegible] conduction velocity in the false tendons

At the extreme periphery of the Purkinje system records of the transmembrane action potential show all gradations between a typical Purkinje fiber action potential and action potentials of ventricular muscle fibers It is reasonable to assume that this finding results from a progressive change in the membrane properties that parallels the gradual change in histologic structure[18] Action potentials recorded from ventricular muscle differ from those of the Purkinje system primarily in that the rate of rise is less the amplitude and duration are somewhat less and there is a steady level of membrane potential during diastole Ventricular fibers are smaller in diameter branch more frequently and conduct at a lower velocity than do Purkinje fibers

Physiologic Basis for Disturbances in Conduction

An attempt to present a detailed description of the physiologic mechanisms responsible for any of the disturbances in A V transmission observed in the clinic certainly is premature On the other hand it is possible to describe the changes in electrical activity that have been observed in association with experimental conduction disturbances and to indicate the extent to which these changes might cause certain electrocardiographic alterations

Disturbances of A V Nodal Transmission

Records from isolated preparations of A V nodal tissue of rabbit and dog heart have shown that most changes in conduction through the

Figure 4 reproduced from Hoffman and Cranefield Electrophysiology of the Heart.[8] By permission of the McGraw Hill Book Company

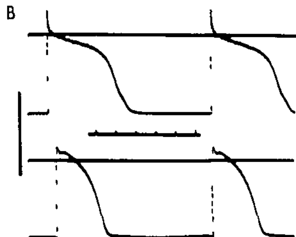


Figure 9

Transmembrane action potentials recorded from single fibers of Purkinje system (upper record) and ventricle (lower record) in an isolated preparation of canine heart Time calibration between records shows intervals of 100 msec vertical bar at left shows voltage calibration of 100 mv Upstrokes of action potentials have been retouched with dashed lines

node and most instances of block are associated with altered electrical activity of fibers at the atrionodal junction [14] A typical example is the delay and block of nodal transmission caused by acetylcholine Action potentials recorded from fibers in the lower node and bundle of His show only those changes that are produced by the acetylcholine induced change in frequency [19] Transmembrane action potentials recorded from nodal fibers at the atrial margin on the other hand are profoundly altered Under the influence of acetylcholine the action potential upstroke becomes slower and more notched and the action potential decreases in amplitude (figs 5* and 6) Often it is replaced by 1 or more small depolarizations that vary in size and to a greater or lesser degree undergo temporal summation Delay and failure of transmission undoubtedly result from these changes however the exact cause of the changes noted is less certain

During complete failure of A V transmission owing to acetylcholine, activity originating in the bundle of His and propagating back to the same nodal fibers elicits an action potential of good amplitude (figs 5 and 6) Moreover the upstroke of this action potential often is free from slurring or notching even when these changes were prominent during normal A V transmission Although direct measurements of threshold are lacking some estimate of the effect of acetylcholine on excitability of the nodal fibers can be obtained from an inspection of records similar to those in figure 6 The small depolarizations recorded during partial block show temporal summation comparison of the level of depolarization caused by such summation which is just sufficient to cause propagation through the node with the level of membrane potential at which the action potential shows an abrupt transition from slow to rapid depolarization suggests that the threshold potential is not much changed by acetylcholine It does not increase the resting potential of fibers in the A V node as it does in the sinoatrial node, this observation at least does not give any positive support to the possibility that acetylcholine causes block by decreasing membrane resistance of nodal fibers The block caused by acetylcholine thus appears to result from the failure of the fibers at the atrial margin of the node to develop an action potential and this failure is associated in some way with asynchronous excitation of these fibers Both the failure of excitation and the asynchronous excitation may be due to the action of acetylcholine on the atrial fibers at the atrionodal junction Action potentials recorded from them are greatly decreased in amplitude during acetylcholine induced block This may be caused by the effect of acetylcholine on potassium permeability which is known to occur in atrial muscle a greatly enhanced K^+ efflux may partly cancel the depolarization caused by an inward Na^+ current [20]

Disturbances of A V transmission caused by premature atrial beats or a rapid atrial rate are associated with somewhat different changes in nodal action potentials At the

*Figure 5 reproduced from Cranefield et al Circulation Research 7 19 1959 [19] By permission of the American Heart Association Inc

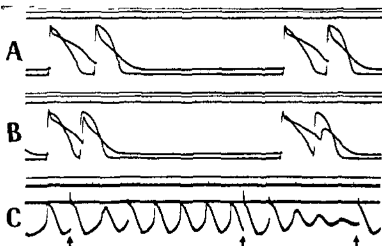


Figure 10

A and B Transmembrane action potentials recorded from an isolated Purkinje fiber–papillary muscle preparation of canine heart Extrasystoles in the papillary muscle (lower trace) excite the Purkinje fiber at various levels of membrane potential during repolarization and elicit either premature action potentials or local responses (last action potentials in B) C Transmembrane action potentials recorded from an isolated preparation of canine Purkinje fibers Marked pacemaker activity has been induced by an excessive concentration of digitalis Extrasystoles (arrows) caused by test pulses contrast markedly in terms of rate of rise and amplitude of the action potential with the action potentials of intrinsic origin

atrial margin of the node the amplitude of the transmembrane action potential is reduced and the rate of depolarization is slowed (fig 7 1) When these changes are extreme records from fibers in the lower node may show a slow steplike depolarization of considerable duration preceding the local action potential (fig 7*B*) During failure of transmission only the graded steplike depolarization is recorded Block caused by agents such as digitalis or quinidine is associated with similar changes in nodal action potentials[15] During block of retrograde impulses that reach the node the failure of conduction most often is localized to the atrionodal junction Also action potentials recorded at different sites within the node are different during normal and retrograde transmission[16] the change is in the initial segment of the upstroke and probably is related to the anatomic arrangement of the various fibers

Delay and Block Within the Bundle of His and Bundle Branches

Experimentally produced conduction disturbances within the bundle of His and bundle branches most often result from 2 factors local differences in action potential duration and/or the presence of slow diastolic depolarization associated with latent pacemaker activity In both cases the failure of normal conduction results directly from the low membrane potential[1] If the transmembrane potential is reduced because of either incomplete repolarization or local pacemaker activity the rate of rise and amplitude of the action potential are decreased (fig 8) The altered action potential may propagate at a reduced velocity or may constitute a purely local response If the reduced conduction velocity permits the adjacent membrane to repolarize completely the slowing of conduction may be localized to a small segment of the conducting system If on the other hand slowing of conduction is caused by diastolic depolarization adjacent areas of membrane will have reached still lower levels of membrane potential and decremental conduction and block may result

Block at the Junction of Purkinje Fibers with Ventricular Muscle

During A V transmission block at the junction of Purkinje fibers with ventricular muscle fibers is unlikely for several reasons The transition from the larger Purkinje fibers to the smaller muscle fibers is gradual and thus there is not an abrupt increase in the area of excitable membrane Also the duration of the ventricular action potential is consider

ably less than that of the Purkinje fibers in adult mammalian hearts (fig 9) Hence the likelihood of a premature impulse reaching the ventricle before it is fully repolarized is reduced However, premature depolarization of ventricular muscle may be delayed or blocked at the junction with the Purkinje system (fig 10 *A* and *B*) and the impulse may propagate at reduced velocity for a considerable distance Although a conclusive experimental demonstration is lacking it is likely that excitation may enter some branches of the Purkinje system and fail to enter others Whether this would cause local re excitation of the ventricle has not yet been determined

Supernormality

The term ' supernormality is used with 2 meanings in descriptions of cardiac excitability it may refer to a reduced stimulus requirement or it may refer to conduction that is either faster than expected or takes place under conditions that might be expected to cause block The cause of both forms of supernormality is clear from studies of transmembrane action potentials [21, 2] Enhanced excitability is found during the terminal phase of repolarization however the action potential elicited at this time is reduced in amplitude and propagates slowly A supernormal phase of conduction is observed in fibers which are partially depolarized or in which there is appreciable diastolic depolarization In such fibers membrane potential reaches its highest value just at the end of repolarization An action potential elicited at this moment will show a higher rate of rise and greater amplitude than responses that occur later during the cardiac cycle (fig 10*B*) Also the larger action potentials will propagate more rapidly and will have a greater safety factor

Acknowledgment

Many of the studies on which this article is based were carried out in collaboration with either Paul F Cranefield or Jackson H Stuckey it is a pleasure to acknowledge the major contributions they have made to these experiments and to many of the concepts that are expressed in this paper

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Calcium Movements in Muscle

By C Paul Bianchi, Ph D

The movements of calcium in muscle have been followed during contraction and contracture to test the hypothesis that the release of calcium from the surface of the muscle membrane during stimulation initiates the contractile mechanism Nitrate ion increases the calcium influx during a single twitch and during potassium contracture and also increases the tension developed The increased entry of calcium during a potassium contracture is transient and not sustained as is the contracture Caffeine which brings about a contracture without depolarization of the membrane and despite the absence of calcium from the medium causes calcium to be released from the muscle

THE PRESENT DISCUSSION of calcium movements in muscle will deal with frog sartorius muscle In the next paper Dr Winegrad will consider recent findings on heart muscle

Calcium has long been proposed as the link between membrane depolarization and contraction [1-4] Of all the physiologic ions that have been injected in small quantities calcium alone causes contraction [5, 6] Sandow in 1952 made a detailed correlation of the kinetics of the sequence of excitatory and mechanical events (fig 1*) At 13 C the rise time of the spike potential is 0 6 msec the time from the peak of the spike potential to the onset of latency relaxation is 1 2 msec and it is during these time intervals that the muscle is mechanically quiescent The time interval until the earliest sign of development of tension the inflexion point of latency relaxation is 3 5 msec and the total time until the onset of tension above the initial tension is 5 4 msec At 25 C the corresponding time intervals are approximately 0 2 msec and 2 5 msec respectively It is during the mechanically quiescent period that 2 processes are occurring (1) membrane depolarization and (2) an intervening process between the peak of the action potential and the beginning of latency relaxation termed the 'spike activation link [2] The size of the spike potential appears to be unrelated to twitch tension for anions that potentiate the twitch e g Br NO_3 I SCN CH_3SO_4 have been shown to have little effect on the spike [3, 7, 8] Hodgkin and Horowicz[9] have found agreement between the threshold of depolarization necessary for mechanical activity during K contracture and the degree of depolarization necessary to initiate the spike potential suggesting that it is the lowering of the membrane potential to a critical level, rather than the height of the spike potential that is concerned in excitation contraction coupling

Under physiologic conditions the conducted action potential of frog sartorius muscle initiates a process whereby contraction occurs Membrane depolarization itself is not the most intimate link in the process for contraction can be brought about without depolarization of the muscle membrane Thus caffeine can cause a contracture without any change occurring in the resting membrane potential [10] Potassium induced contracture can fail to occur even though the muscle membrane is depolarized if external calcium is removed [11] Csapo has shown that treatment of the turtle retractor penis muscle with NaI can bring about greater tension development with smaller membrane depolarization [1] Hodgkin and Horowicz have demonstrated that replacement of Cl with NO_3 can lower the threshold of membrane depolarization necessary to bring about a contraction [13] All of these findings point to another intervening process between membrane depolarization and the initiation of mechanical activity

Studies on calcium movements in muscle

From the Institute for Muscle Disease New York New York

Supported by a grant from the Muscular Dystrophy Associations of America Inc

Figure 1 from Sandow Yale J Biol & Med 25 176 1952 By permission of the journal

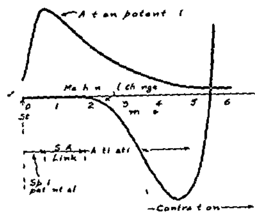


Figure 1

Temporal correlation of excitation and mechanical events during latent period of frog sartorius muscle at 13 C according to Sandow (From Sandow [5])

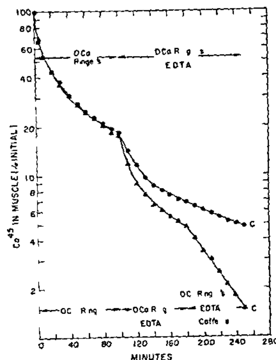


Figure 2

The effect of edathamil (EDTA) and caffeine on the washout of Ca^{45} from a muscle previously soaked for 5 hours in Ca^{45} Ringer's solution At 100 minutes 0 001 M EDTA is added to the medium bathing both C and C An immediate increase in Ca^{45} release occurs which tapers off after 110 minutes At 180 minutes 0 005 M caffeine is added to muscle C causing a sustained increase in the release of Ca^{45} from the muscle

conducted by Shanes and Bianchi provide evidence that an increase in calcium influx during depolarization by an action potential or by an increase in extracellular K is part of the intermediate process between events in the muscle membrane and mechanical activity of the contractile proteins Table 1 shows that calcium influx in unstimulated muscle is 0 094 micromicromoles/cm sec Stimulation of the muscle causes additional calcium to enter the muscle which amounts to 0 2 micromicromoles/cm twitch If one assumes that calcium enters the muscle fibers at a high rate immediately upon depolarization and continues to enter during the period of mechanical quiescence (1 0 msec at 25 C) then the resting influx rate of calcium would have increased from 0 1 micromicromoles/cm sec to 200 micromicromole/cm sec an increase of approximately 2 000 If the calcium were considered to enter during the period from membrane depolarization to the appearance of initial tension development (2 5 msec) then the increase would still be 800 fold

The amount of calcium entering per twitch and the twitch height is increased 60 per cent by replacing the chloride of Ringer's solution with nitrate Under these conditions nitrate has no effect on the unstimulated influx of calcium showing that nitrate affects only the calcium entering during a twitch Nitrate has been shown to prolong the active state [14 15 16] which would account for the increased twitch height that is observed in nitrate Ringer's solution A transitory increase in the ionized calcium level of the muscle fibers brought about by the increased amount of calcium entering per twitch could account for the prolongation of the active state In keeping with this hypothesis superficial muscle fiber sites have been shown to bind about 0 1 micromole/Gm of calcium in nitrate Ringer's solution [17] The increased binding of calcium and the increased influx during stimulation are in agreement with the suggestion of Shanes[4] that the enhancement of the twitch height when other halogens or nitrate replaces chloride may be due to an improved binding of

Table 1

Calcium Influx in Frog Sartorius Muscle

Condition	Influx
Unstimulated	
Ringer's solution	0 094 μμmole/cm sec
Ringer's nitrate solution	0 108 μμmole/cm sec
Stimulated	
Ringer's solution	0 [illegible]0 μμmole/cm² twitch
Ringer's nitrate solution	0 32 μμmole/cm² twitch
Ringer's solution + 20 mM KCl	0 1[illegible]4 μμmole/cm² sec
Ringer's solution + 80 mM KCl (after relaxation)	0 0[illegible]6 μμmole/cm² sec
Ringer's solution + 80 mM KCl (initial depolarization)	38 μμmole/cm²
Ringer's nitrate solution + 80 mM KNO_3	60 μμmole/cm²

calcium to the membrane, which in turn contributes to enhanced entry of calcium during stimulation.

Depolarization of the sartorius muscle by 20 mM K, a level just below the threshold for contracture, leads to a sustained increase in calcium influx (table 1). Calcium influx measured in the presence of 80 mM K after relaxation from the induced contracture is smaller than calcium influx in 20 mM K and almost equal to influx in unstimulated muscle. The entry during initial depolarization in 80 mM K amounts to 38 micromicromole/cm²[18]. The presence of nitrate increases the amount to 60 micromicromole/cm², which is in keeping with the potentiated contracture that is observed.[19] The large transient increase in calcium influx during initial depolarization is consistent with the rapid increase in tension during the first second of KCl contracture observed by Hodgkin and Horowicz.[9] The relaxation of the phasic muscle during maintained KCl depolarization can be interpreted as a failure to sustain the high rate of calcium influx. Shanes[6] has shown that a high rate of calcium entry persists in the slow fibers of the frog rectus abdominis along with the sustained potassium contracture. Thus in fast fibers both the increased influx of calcium and the contracture are transient during K depolarization, whereas both are sustained in slow fibers during K depolarization.

Contracture brought about by caffeine has important differences from potassium induced contracture. Potassium contracture is not sustained and is associated with a transitory increase in calcium influx during the initial membrane depolarization. Removal of external calcium prevents potassium contracture.[11] In contrast, caffeine causes a sustained contracture in frog sartorius without membrane depolarization and in the complete absence of external calcium. The site of caffeine action is on the membrane. Axelsson and Thesleff[10] have shown that only caffeine applied externally to the membrane results in a contracture, while caffeine applied by injection to the muscle interior is without effect. It has also been shown that caffeine markedly increases calcium outflux and influx.[18] Figure 2 shows that even after prior treatment of frog sartorius muscle with edathamil (EDTA), which can remove some superficial bound calcium as well as calcium in solution, caffeine causes a marked increase in calcium outflux, suggesting that caffeine can bring about the release of calcium from membrane sites and perhaps sarcoplasmic reticulum sites, which in turn results in contracture. The increased calcium outflux may therefore reflect the freeing of bound calcium, which would raise the intracellular calcium ion content and thus induce contracture without the necessity of external calcium or a membrane depolarization.

The release of calcium during stimulation has been observed by Woodward[1] and confirmed by Shanes and Bianchi.[2] Figure 3 clearly demonstrates the release of calcium during tetanic stimulation. Potassium contractures, both isotonic and isometric, increase calcium outflux (fig. 4). The increased outflux during tetanic stimulation is not sustained and the minimum calcium released per twitch is about the same as the amount taken

up per twitch viz 0 2 micromicromole/cm

Potassium contracture results in a rapid release of calcium which is at about double the base line rate even after 10 minutes (fig 4) The increased influx and outflux of calcium in frog sartorius observed with tetanic stimulation or potassium contracture may reflect the same basic process such as freeing of calcium from the surface supported by the rapid release of calcium during tetanic stimulation Two other possible explanations for the increased influx and outflux are (1) a spatial separation in which different sites of the membrane involve calcium influx and outflux and (2) a temporal separation of the two fluxes

From the foregoing it is evident that calcium influx into the muscle fiber is related to mechanical activity in 2 ways The enhanced twitch height and contracture in nitrate Ringer s solution is correlated with a larger influx of calcium and there is a temporal relationship between the duration of increased calcium influx and of mechanical activity Potassium contractures and a high rate of calcium influx are transitory in phasic muscles while in slow fibers potassium contractures are sustained as is the high rate of calcium influx The manner in which calcium brings about activation of the contractile mechanism is still unknown although from experiments on model systems there appear to be 2 possible modes of action One would be the inhibition of the relaxing factor system thus allowing contraction to take place with relaxation occurring as the ionized calcium is removed the other would be by a direct action of calcium on actomyosin Weber[3] has shown that calcium in a concentration of 10^{-4} M which would be equivalent to 5 × 10^{-5} M ionized calcium gives a maximum activation of the highly purified actomyosin ATPase system and also maximum superprecipitation of actomyosin with 2 mM Mg ATP In the absence of added calcium no superprecipitation could be measured and the ATPase activity was reduced to 20 per cent of the maximum activity There is however a large discrepancy between the

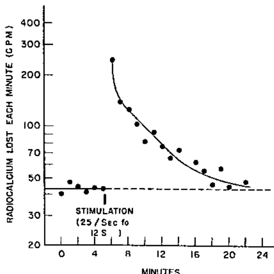


Figure 3

The release of Ca^{45} during a brief tetanic stimulation from frog sartorius muscle that has been previously soaked in Ca^{45} Ringer's solution Sample collections of the medium bathing the muscle were made at minute intervals in order to obtain the true time course of calcium release

amount of calcium needed for either inhibition of the relaxing factor system or activation of the actomyosin ATPase system If the calcium entering per twitch (0 2 micromicromole/cm ^{2}) were uniformly distributed in the muscle fiber water then the final concentration of ionized calcium would be 10^{-7} M which is too small a concentration by a factor of 100 for both the relaxing factor system and the actomyosin ATPase The discrepancy is still larger when the number of calcium ions that enter in relation to the actomyosin concentration of muscle is considered If one estimates 100 mg of actomyosin for every gram wet weight of muscle and a molecular weight of 500 000 then the actomyosin concentration of frog muscle would be approximately 2 × 10^{-4} M as compared to 10^{-7} M for calcium Much more ionized calcium would be needed than can be accounted for by the calcium entering per twitch suggesting perhaps that the initial entry of calcium from the membrane can bring about a further release of calcium from binding sites localized in the sarcoplasmic reticulum.

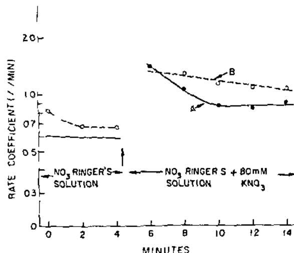


Figure 4

Comparison of the time course of calcium release during isotonic (A) and isometric (B) contracture The calcium released is plotted as a rate coefficient i e the percentage of the exchangeable calcium in the muscle being released during the 2 minute collection intervals The contracture occurs when 80 mM KNO_3 is added to the NO_3 Ringer's solution

Thus calcium influx is markedly increased during a muscle twitch and potassium contracture in frog sartorius muscle Nitrate ion which potentiates both the twitch and potassium contracture also increases the entry of calcium under these conditions The caffeine induced contracture may be accounted for by an increase in the ionized calcium level in the muscle fiber brought about by a direct action of caffeine on calcium binding sites in the membrane and perhaps those located in the sarcoplasmic reticulum

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The Possible Role of Calcium in Excitation-Contraction Coupling of Heart Muscle

By SAUL WINEGRAD, M.D.

The transfer of Ca^{45} at rest and during contraction has been measured in isolated guinea pig atria. During contraction the rate of transfer increases considerably. The increment in the uptake of calcium by the cells of the atria during contraction is closely correlated with the strength of contraction. This relationship is maintained at different frequencies of stimulation and at different concentrations of external calcium.

THE IMPORTANCE of calcium in the contraction of heart muscle has been known since the experiments of Ringer.[1] Early work[2, 3] focused primarily on the dependence of the strength of contraction on the concentration of calcium ions in the bathing solution. More recently an antagonism between sodium and calcium ions at the cell surface has been inferred from the observation that the effects of a decrease in extracellular sodium concentration and an increase in extracellular calcium concentration on twitch tension are similar.[4] The observation that withdrawal of calcium ions from the bathing solution caused rapid disappearance of mechanical but not of electrical activity of isolated heart muscle implicated the calcium ion as the excitation contraction link.

In a series of studies on frog ventricular strips Niedergerke and Luttgau[6] showed that changes in external sodium and calcium concentrations very rapidly altered the characteristics of potassium induced contracture and that these changes in contracture tension could be produced even after the initial potassium depolarization was complete. Figures 1 to 3* are taken from the work of Niedergerke and Niedergerke and Luttgau.[6] Figure 1a and b shows the effect of replacement of sodium in the bathing solution at the beginning of a potassium induced contracture. In figure 2a† the ventricle was depolarized by 100 mM potassium in the presence of 10 mM calcium, while in figure 2b the initial depolarization occurred in 0 Ca, 100 mM potassium but after 90 seconds the solution was changed to one containing 10 mM Ca, 100 mM potassium. The effect of the calcium is clearly not limited to processes accompanying depolarization. Similarly the effect of decreased sodium concentration can occur after depolarization (fig 3). Electrical measurements eliminated increased depolarization as an explanation for the increases in tension seen with elevated calcium or depressed sodium concentrations.[8]

By use of radioisotopes Niedergerke and Harris[9] demonstrated that the changes in extracellular sodium and potassium concentration associated with increased twitch tension were accompanied by increased uptake of Ca^{45} by the resting tissue (fig 4‡) and by the tissue in potassium-contracture[10] (fig 5§).

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The results described here were first presented by the author at the Fall Meetings, American Physiological Society, 1960 (Winegrad, S.: Physiologist 3: 1 9, 1960) and are to be presented in detail (Winegrad, S., and Shanes, A. M.: Calcium transfer and contractility in guinea pig atria. In preparation.)

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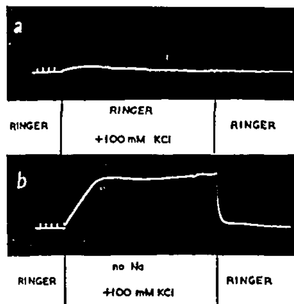


Figure 1

Dependence of contracture on sodium chloride concentration Contractures always induced after a 30 min period of equilibration in Ringer's fluid during which strip was stimulated at a constant rate Ringer's fluid and potassium rich solutions contained 0.5 mM calcium chloride throughout the experiment Diameter of strip 0 35 mm (a) Contracture in the presence of 100 per cent sodium chloride induced by adding 100 mM potassium chloride to Ringer's fluid (b) Contracture induced by adding 100 mM potassium chloride and simultaneously replacing the sodium chloride content of Ringer's fluid by sucrose (From Niedergerke and Luttgau [6])

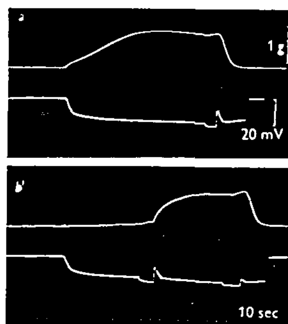


Figure 2

Effect of Ca on a depolarized strip diam 1 1 mm In both records upper traces tensions lower traces depolarizations The records started after soaking the strip in Ca free Ringer's solution for 10 min (a) Application of 10 mM Ca 100 mM KCl solution, (b) application initially of a Ca free 100 mM KCl solution 90 sec later 10 mM Ca was added to this solution causing an immediate rise of tension (From Niedergerke [7])

These studies dealt primarily with rapid changes in calcium transfer, a distinction between changes occurring at the cell surface and in the cell interior based on a difference in time constants of calcium transfer was not clear

The demonstration by Bianchi and Shanes[11] of an increased uptake of calcium during contraction of skeletal muscle fibers added substantially to the data implicating calcium as the excitation contraction link To test the possibility that excitation in myocardial cells is coupled with contraction by a movement of calcium into the cell during depolarization a series of experiments comparing calcium uptake by guinea pig atria at rest and under several different conditions of contraction was conducted Radioisotope technics similar to those of Bianchi and Shanes[11] were used Tensions were measured with conventional strain gauges Left atrial appendages from young guinea pigs were used because the tissues are thin stable over long periods of time and devoid of spontaneous rhythm

Calcium influx was determined by soaking the atria for 15 minutes in Ca^{45} solution and measuring the amount of Ca^{45} remaining in the tissue after the extracellular and loosely bound surface Ca^{45} had been washed out in nonisotopic solution A correction was made for the intracellular Ca^{45} lost during the washout To eliminate the error introduced by the presence of damaged tissue the edges of the atria cut during dissection were removed from the undamaged tissue at the end of the experiment and their radio

Table 1
Relationship of Ca^{45} Uptake to Twitch Tension

Ca mm U/ml	Rate b/min	Rel U Ca	Rel tension	N
1 ɔ	15	3 ± 1ɔ	39 ± 9	ɔ
2.50	6	30 ± 9	38 ± 5	6
2.50	1ɔ	9ⁿ ± 15	86 ± 9	14
ⁿ 0	30	100 ± 14	100	3
3 ɔ	6	5 ± 3	ɔ8 ±	4
3 ,ɔ	15	106 ± 14	10ɔ ± 6	11

Values ± 1 standard error of mean

activity counted separately The effect of contraction on influx was measured by stimulating the muscles during the last 10 of the 1ɔ minutes in Ca^{45} Krebs solution and calculating the difference in Ca^{4} content between the stimulated and unstimulated muscles

The resting influx at external calcium concentrations of 1 2ɔ mM 2 50 mM and 3 7ɔ mM was 0 008 ± 0 0010 0 014 ± 0 0012 and 0 022 ± 0 0046 micromicromole cm sec In figure 6 the influxes are plotted against the external calcium concentration The data can be best approximated by a straight line that when extrapolated misses the origin by a statistically insignificant amount It would appear therefore that in the resting muscle cell influx of calcium is a function of the external calcium concentration The resting outflux in 2 ɔ mM calcium calculated by standard desaturation studies was approximately equal to the influx suggesting that in the resting atria no net movement of calcium was occurring

When the atria contracted the calcium influx increased considerably the total calcium⁴ content of the stimulated tissue at the end of the experiment rose to as much as 1ɔ times that of the resting controls Under these circumstance the increment in calcium influx associated with each beat was 0 ɔ⁷ micromicromole cm surface area Contraction was not accompanied by an increase in calcium uptake by the cut edges of the tissue which themselves did not contract

It was of interest to determine whether

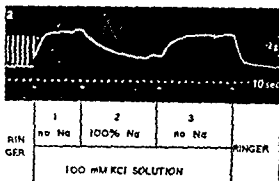


Figure 3

Reversibility and specificity of the action of sodium ions Ringer's fluid and potassium rich solutions contained 2 mM calcium chloride throughout the experiment Diameter of strip 0 15 mm (1) Contracture induced by adding 100 mM potassium chloride to Ringer's fluid and simultaneously replacing sodium chloride iso osmotically with sucrose (2) reapplication of sodium chloride, (3) replacement of sodium chloride by sucrose (From Niedergerke and Luttgau⁸)

any correlation existed between the size of the calcium influx per beat and the twitch tension Table 1 consists of the results of experiments performed to study the effect of different external calcium concentrations and different frequencies of contraction on both the calcium uptake and the strength of contraction Calcium uptake per beat and twitch tension for each condition are expressed in per cent relative to the values at a contraction frequency of 30/min in 2 5 mM calcium the latter having been arbitrarily assigned a value of 100 per cent

The uptake per beat of atria contracting at 6/min in 2 ɔ0 mM calcium is 30 per cent of that of the atria beating at 30/min ($p<0.0ɔ$) The percentage increase in uptake per beat associated with this change in the rate of contraction does not differ significantly from the percentage increase in twitch tension When the frequency is changed from 1ɔ/min to 30/min no significant increase in either twitch tension or uptake per beat occurs Thus in these experiments both calcium influx and contractility apparently exhibit parallel saturation properties

Muscles stimulated at 1ɔ/min in 1 2ɔ mM

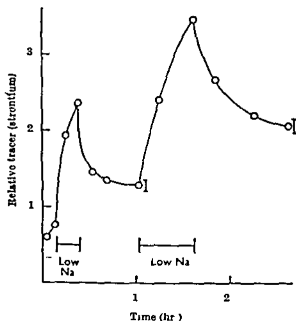


Figure 4

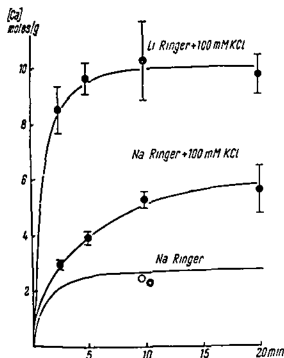
The effect of replacing the sodium chloride of Ringer's fluid by sucrose on the uptake of strontium 89 tracer in heart tissue Relative amounts of strontium 89 tracer in a ventricle strip during exposure to tracer solutions which contained either 117 5 mM sodium chloride or 5 mM sodium chloride + 202 mM sucrose The two solutions had identical concentrations of potassium chloride (2 mM) and of tracer Sr^{89}–Ca (1 mM) mixture Vertical bars indicate standard error of the measurement of radioactivity Sr^{89} is used as an indicator of Ca movement (From Niedergerke and Harris [9])

calcium contract about as vigorously as the atria beating at 6/min in 2 50 mM calcium Despite the fact that two important conditions have been changed in opposite directions the relative uptake per beat and relative tension are still closely correlated An increase in external calcium concentration from 1 25 mM to 3 75 mM increases twitch tension almost 3 fold ($p<0\ 05$) and calcium uptake per beat is increased proportionately An increase in the calcium concentration from 2 5 mM to 3 75 mM is associated with proportionately smaller increases in uptake and twitch tension but in this comparison both changes are of questionable significance

When a plot is made of relative twitch tension vs relative uptake per beat using all the data obtained from different external calcium concentrations and different rates of stimulation (fig 7), a consistent correlation between the 2 parameters exists A straight line closely fits the data and its failure to pass through the origin is not statistically significant

These data demonstrating the increment in calcium influx associated with contraction are consistent with the hypothesis that calcium movement into the cell with depolarization couples excitation with contraction They suggest also, that such a link is a factor in determining the strength of the twitch

In an analysis of the increment of calcium influx associated with contraction one may consider (1) the source of this calcium (2) the mechanism by which it enters the cell (3) the time in the cardiac cycle during which the added calcium enters the cell and (4) the possible mode of action inside the cell Niedergerke s data[9, 10, 1] suggest that superficial sites in the resting cell bind calcium and that certain changes in the composition of bathing solution which produce increased twitch tension are associated with greater binding of calcium by the resting tissue Moreover, experiments already mentioned show that similar changes in the composition of the bathing solution during a maintained potassium depolarization are rapidly followed by changes in contracture tension An additional pertinent observation is that of Weidmann [13] who demonstrated in turtle ventricle that a sudden increase in the concentration of calcium in the extracellular space during the initial stages of a twitch produced a more rapid rate of tension development and a greater peak tension than occurred at the lower calcium concentration In addition the increase in calcium concentration was accompanied by a shortening of the action potential A hypothesis that incorporates these data with the observed correlation between the rate of calcium influx during a contraction and the size of the contraction would be the following the calcium that enters the cell with contraction comes from



a

Figure 5

Effect of K depolarization of Ca uptake of frog ventricular strips The difference between the lower two curves shows additional Ca uptake due to 100 mM KCl Na Ringer Upper curve shows Ca uptake in absence of Na but with additional 100 mM KCl Hollow circle and double circle represent two experiments showing Ca uptake in Ringer's solution made hypertonic by added 180 mM sucrose or added 100 mM LiCl (From Niedergerke [10])

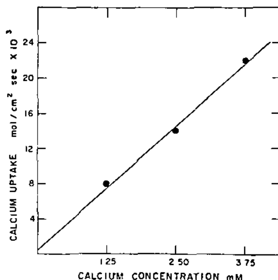


Figure 6

Calcium uptake of resting guinea-pig atrial appendage is plotted against extracellular calcium concentration

superficial sites and from the extracellular fluid the calcium that enters the cell from the extracellular fluid during depolarization passes through the same superficial sites to which calcium was bound in the resting state

Weidmann's data[13] suggest that the increment in calcium influx occurs during the depolarization In support of this conclusion are the observations that the increased heart rate is associated with a shorter diastole a shorter action potential but an increased calcium influx per beat and an increased rate of rise of tension during the twitch [14-16] If the influx of calcium is quantitatively related to twitch tension it might be expected that a greater increment during a shorter action potential would produce a more rapid rise in tension If the added calcium does enter the cell during the action potential (measured to be 150 msec) then calcium influx during depolarization may exceed resting influx by as much as 250 fold

Certain possible relations of the calcium influx to contraction became apparent with a quantitative consideration of the data If the molecular weight of myosin is about 500 000 [17] and its concentration in heart muscle is equal to that in skeletal muscle (taken to be 7 6 per cent of wet weight[18]) then each gram of heart contains $1\ 5 \times 10^{-4}$ mmoles of this protein The maximum increment in calcium influx measured in these experiments was $0\ 6 \times 10^{-6}$ mmoles/Gm The ratio of the number of calcium ions entering the cell during contraction to the number of myosin molecules already present is about 1/250 A similar ratio results if a comparison is made of the calcium influx to the amount of actin or tropomyosin in the muscle (again assuming that the concentration of actin and tropomyosin in heart muscle is similar to that in skeletal muscle) It is unlikely that only 0 4

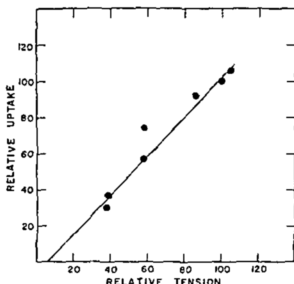


Figure 7

The relative calcium uptake per beat of guinea pig left atrial appendages contracting at 6/min 15/min 30/min in 1 25 mM 2 50 mM and 3 75 mM Ca–Krebs solution is plotted against the relative twitch tension produced All values are expressed relative to calcium uptake and twitch tension at 30/min in 2 5 mM Ca–Krebs solution

per cent of the contractile protein is shortening during the strongest contractions of the isolated atrium Therefore if calcium does initiate the contraction each ion must ultimately have an effect on many actin myosin units One of the reactions in the contractile process though not necessarily the one involving calcium must involve either a chain reaction or the interaction of 1 molecule or ion with as many as 250 molecules or units The latter could occur by an enzymatic reaction or by a specific type of molecular alignment in which one molecule is in close association with many contractile units

With respect to this quantitative relationship of calcium taken up during contraction *to actomyosin it is interesting to note the* calculation of H E Huxley[18] that each thick filament in skeletal muscle contains about 400 myosin molecules If a similar condition exists in the heart the ratio of calcium ions taken up in a maximal contraction to the number of thick filaments is 1 6 1

If the calcium that enters the cell with contraction is assumed to achieve immediate uniform distribution *in the cell water* the concentration would be 1 2 × 10^{-6} M Any non uniform distribution would produce regions within the cell of higher ' calcium concentrations ' This value of ionized calcium inhibits the relaxing factor activity in vitro[19] Ebashi has further shown that the relaxing factor system binds calcium tightly and that procedures which decrease calcium binding proportionately decrease relaxation activity He has demonstrated that other calcium chelating agents have relaxation activity pro*portionate to their ability to chelate calcium*[20] It is possible therefore that the calcium that enters the cell during excitation inhibits the relaxing system and thereby initiates contrac*tion or that it activates* contraction relaxation occurring by the removal of the calcium by the relaxing system

Acknowledgment

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Cardiac Active Principles in Blood Plasma

By Stephan Hajdu M D, and Edward Leonard, M D

A study of isolated frog hearts suggests that the amphibian heart is provided normally with a substance that maintains contractility and that disappears slowly from the heart during perfusion with saline solution A search for substances that might fulfill this role in the frog heart has led to the isolation of many materials that have some degree of cardio tonic activity but no conclusion can yet be reached about which if any of these substances is present in the intact frog heart The search has led however to the discovery of 2 substances of mammalian origin that are as potent as the cardiac glycosides with respect to their inotropic action on frog heart One of these is a phospholipid called lysolecithin the other is a system of 3 plasma globulins called cardioglobulin A B and C ' The concentration of cardioglobulin C in man is increased in essential hypertension and aortic stenosis 2 unrelated conditions that have in common the development of increased left ventricular isometric tension in systole Conversely cardioglobulin C is decreased in a group of patients with idiopathic congestive heart failure The discovery of these substances is relevant to the question whether isolated mammalian cardiac tissue becomes hypodynamic in physiologic saline because of the loss of a system that helps maintain normal myocardial contractility We have noted that most of the studies on isolated strips of mammalian cardiac tissue fail to answer this question since the strips were probably hypodynamic because of impaired oxygenation or nonphysiologic saline media Studies from our laboratory indicate that a slow decline in contractility on prolonged washing does occur in isolated mammalian heart tissue despite good oxygenation and a normal environment with respect to inorganic ions This can be prevented or reversed by perfusion with mammalian plasma Along similar lines it is of interest that although the decline of performance characteristics of in situ mammalian hearts may be due to many factors the decline can be prevented by perfusing the coronary system of the in situ heart with blood from a healthy donor animal The problem then has 2 aspects On the one hand we must discover the physiologic significance of the potent glycoside like substances already isolated from mammalian tissue on the other we must investigate the beneficial effects of plasma on heart strips or of donor dog blood on in situ hearts Do these actions occur because of an effect on myocardial metabolism or do they come about because of a plasma substance that enhances myocardial contractility directly?

A WIDE VARIETY of experience has led to the conclusion that blood plasma has a beneficial effect on cardiac contractility Thus cardiac function declines when hearts are bathed in artificial saline media and improves when fresh whole blood or plasma is added This effect was first noted by Ringer in 1885 [1] Recent interest in this phenomenon stems from two different lines of interest On the one hand now that much information has been obtained about the contractile protein of muscle and about the metabolic events that yield energy for contraction it is appropriate to consider the problem of how the force of muscle contraction is regulated The same question about the regulation of muscular contraction presses to the fore in the field of cardiovascular research We may mention as examples the abnormal increase in arteriolar tone in essential hypertension, the congestive heart failure that occurs in a number of patients in the absence of any known structural or inflammatory disease of the myocardium and the cardiovascular collapse that often supervenes in patients subjected to a prolonged period of extracorporeal circulation All these instances are characterized by abnormalities of involuntary muscle function despite the absence of any gross anatomic or metabolic defects that might be invoked as causes of the disturbance In this lecture we will review some experimental situations in which alterations in cardiac function might be due to the addition or depletion of cardiac active substances of biologic origin

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We shall begin with the isolated amphibian heart The decline of myocardial contractility that occurs when hearts are bathed in artificial media was studied in detail by A J Clark in 1913 he said that excised frog hearts after perfusion for a few hours pass into a hypodynamic state in which both the force of contraction and the rate of conduction are markedly impaired He concluded that the development of a hypodynamic state was associated with the loss of some essential substance from the frog heart which was washed away slowly by prolonged contact with large volumes of saline solution

There are several features about the hypodynamic heart that we would now like to enumerate First we may note that immediately following excision and immersion in saline a slow decline in contractility begins that continues steadily for a number of hours The rate of decline depends to some extent on the thoroughness of the washing that is to say on the volume of perfusing solution and frequency of exchange The first signs of the decline cannot be detected at high rates of stimulation but can be observed in the form of decreased twitch tension at lower frequencies It should be emphasized that this decline occurs despite an environment that is ideal with respect to inorganic ions and oxygenation Unlike mammalian heart muscle the frog heart has no coronary system and depends for oxygenation on the movement of blood through the sponge like network of muscle cells that constitute the ventricular wall It is therefore no problem for oxygenated Ringer solution to perfuse the various parts of the frog ventricle adequately Not only is there no impairment in oxygenation but it is likely that the development of the hypodynamic state occurs in the absence of any decrease of high energy phosphate Although this has not been studied in frog ventricle Furchgott and de Gubareff[3] have shown that the development of the hypodynamic state in guinea pig atria is not associated with any change in the concentration of high energy phosphate compounds in the atrial muscle It would appear therefore that the hypodynamic state in frog heart muscle is not due to a defect in the supply of phosphate energy to the contractile protein For a more detailed discussion of the question of whether the defect in experimental hypodynamic states is due to a defect in energy supply or energy utilization see reviews by Hajdu and Leonard[4] and by Wollenberger[5]

We may now consider the substances that can restore frog heart contractility to the original level Actually a very great number and variety of substances of plasma origin can improve the hypodynamic heart Ringer concluded that the plasma activity resided in a nondialyzable fraction Clark found that a positive inotropic effect could be obtained not only with soaps, such as sodium oleate but also with various phospholipids and even amino acids Other surface active substances such as bile acids also improved contractility In addition beneficial effects have been obtained with adrenalin[6] adenosine triphosphate (ATP)[7] and with pharmacologic concentrations of certain steroids such as deoxycorticosterone and progesterone[7] A great many investigators have worked in this field and the interested reader can find more references in the review by Amberson[8]

Most of the substances we have mentioned are capable of bringing the contractility of the hypodynamic frog heart back to normal if they are added to the bathing solution in sufficient concentration Their effect can be appreciated by reference to figure 1 in which twitch tension is plotted on the ordinate and the time interval between stimuli is plotted on the abscissa The twitch tension of both the fresh heart and the washed hypodynamic heart varies with the frequency of stimulation Comparison of these curves shows that whereas the twitch tension of the hypodynamic heart approaches that of the fresh heart at high frequencies at intermediate frequencies fresh heart is capable of developing much greater twitch tension than is the hypodynamic heart The beneficial substances we have discussed above cause the hypodynamic curve to shift back toward the fresh heart curve

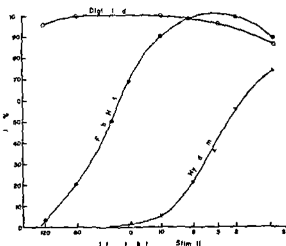


Figure 1

Effect of prolonged saline perfusion on isolated frog hearts

In one respect however the hypodynamic frog heart which is improved by the addition of these materials is still different from a normal heart on subsequent washing the hypodynamic state develops very rapidly This is in marked contrast to the slow and gradual decline in contractility that occurs with a fresh heart It seems reasonable to postulate that the development of the hypodynamic state in the frog heart is due to the slow loss of some material from the muscle One would suspect that it is present in low concentrations in frog blood and that an appropriate cardiac content is maintained because of the constant perfusion and the high affinity of the material for the heart If so perfusion of a hypodynamic frog heart with a large volume of normal frog plasma should result in the slow accumulation of the material so that once again the heart would become hypodynamic only after prolonged washing

Recently a material that may fulfill these conditions has been isolated [9] It is a phospholipid called ' β palmitoyl lysolecithin, which can be found in small concentrations in mammalian plasma It has been isolated from adrenal medulla and may be rather widely distributed in the chromaffin system Although this substance is present in mammalian plasma in concentrations too low to affect the hypodynamic frog heart immediately it becomes bound to frog cardiac muscle so that if a frog heart is perfused with successive changes of serum a significant amount of lysolecithin gradually accumulates And in contrast to the other substances discussed after exposure to this material the restored heart becomes hypodynamic only gradually in the course of prolonged washing Attempts have not yet been made to isolate lysolecithin from frog tissues If it is found in frog plasma it would be reasonable to postulate that lysolecithin is the naturally occurring substance that is slowly washed away from the frog heart during the development of the hypodynamic state

Lysolecithin differs in another way from substances previously shown to restore the contractility of the hypodynamic frog heart This can be appreciated by referring once again to figure 1 which shows how twitch tension varies with the interval between stimuli Lysolecithin like the cardiac glycosides can cause maximal twitch tension even at very low rates of stimulation which is represented in the figure by the nearly horizontal line at the top of the graph In higher or toxic concentrations the glycosides or lysolecithin induce systolic arrest or contracture a phenomenon that is not seen with the other substances we have been discussing Their positive inotropic effect is therefore profoundly greater than that of any of the other substances Recently we have isolated from mammalian plasma a cardiotonic protein system of great potency that is comprised of three globulins that have been called cardioglobulin A B and C [10] The effect of cardioglobulin on the frog heart in a concentration comparable to that found in normal human plasma is similar to that of a nontoxic concentration of cardiac glycosides (This protein system also causes constriction of the peripheral vasculature of the frog in a Trendelenburg preparation and therefore the vasoconstrictor globulin studied by Sakai and Hiramatsu many years ago may have been cardioglobulin [11])

A new question of great interest now arises as a result of these studies of the hypodynamic

amphibian heart The discovery of 2 inotropic systems lysolecithin and cardioglobulin with cardiotonic potency comparable to that of the cardiac glycosides indicates that we must determine whether either of these systems is important in the maintenance of normal myocardial contractility in the mammal In this regard the plasma concentration of cardioglobulin in various clinical states is of interest We have compared the plasma concentration of cardioglobulin C in normals in patients with aortic stenosis and in patients with essential hypertension In both aortic stenosis and essential hypertension 2 conditions characterized by increased left ventricular isometric tension in systole the concentration of cardioglobulin C is significantly increased above normal[1, 13] This is consistent with the idea that cardioglobulin could be a naturally occurring cardiotonic system that is increased in the hyperdynamic states noted The increased cardioglobulin seems to be related to the increased pressure developed by the left ventricle and not simply to increased work since in aortic insufficiency (characterized by increased stroke work without an increase in pressure) cardioglobulin is normal The concentration of cardioglobulin has also been measured in various types of cardiac failure and it has been found that values for patients with failure secondary to valvular disease (aortic or mitral insufficiency) are normal In contrast about half of the patients with cardiac failure secondary to idiopathic myocardial disease appeared to fall into a separate population with extremely low values of cardioglobulin[1, 13] The question whether the myocardial failure of this group may be caused by the observed cardioglobulin deficiency cannot be answered at this time

The questions about cardioglobulin raised by these clinical correlations lead us to consider studies on experimentally induced hypodynamic states in mammalian heart muscle Investigations on surviving strips of mammalian heart received considerable impetus when Cattell and Gold introduced the cat papillary muscle preparation for the study

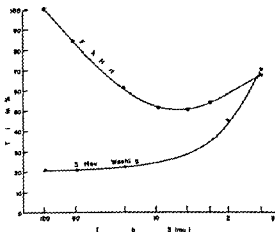


Figure 2
Effect of prolonged saline perfusion on rat right ventricle strips

of the action of cardiac glycosides[14] Since that time a great variety of substances of mammalian origin have been found to increase the contractility of isolated cat papillary muscle including phospholipids[15] amines[1] amino acids[1] serum albumin[16, 37] a dialysate of serum albumin[17] and various adrenal steroids[18] The substances studied have been isolated not only from serum[16, 17] but also from fractions of dried spleen[18] and from liver[1]

In attempting to evaluate these various results it is pertinent to recall that the contractility of the isolated frog heart in saline declines slowly and steadily over a period of hours despite normal energy metabolism and ionic environment suggesting that a substance which maintains normal contractility is gradually washed away from the heart Does a comparable situation exist in the case of mammalian hearts? Unfortunately the studies under consideration cast no light on this question because the comparable experimental conditions do not exist in the mammalian heart preparations The mammalian tissues studied were hypodynamic because of abnormally low calcium or bicarbonate concentration in the extracellular medium in some cases and in others it is probable that impaired oxygenation caused the hypodynamic state In contrast to the frog heart

which has no coronary system oxygenation of dense mammalian ventricular tissue is difficult to accomplish once the coronary circulation is interrupted Because of the difficulty in oxygenation we would not expect cat papillary muscle stimulated at rapid rates to survive without some metabolic abnormality Tanz for example has recently published photomicrographs showing severe histologic damage in cat papillary muscle stimulated at 1 per second at 37 C for 6 hours[19] Therefore although a great variety of substances of biologic origin have been found to improve the performance of mammalian heart preparations it is difficult to judge the physiologic significance of these findings and none of the studies provides an answer to our question about whether there is a cardiotonic material bound to mammalian cardiac muscle that is lost when the tissue is removed from its normal environment

Some recent results of our own which are addressed to this problem can be seen in figure 2 The studies were made on thin strips of right ventricle from very young rats and the graph shows twitch tension plotted as a function of the interval between stimuli The upper curve represents the normal pattern found in freshly prepared rat ventricle After 3 hours of washing in Krebs bicarbonate solution the lower curve is obtained with considerably lower twitch tension over a wide frequency range This can be reversed with digitalis or mammalian plasma and the original decline can be prevented if the strip is maintained from the beginning in mammalian plasma In general it appears that mammalian cardiac muscle strips immersed in an environment which is ideal with respect to inorganic ions and oxygenation becomes hypodynamic on prolonged perfusion and it is probable that this decline in contractility can be prevented if heart strips are maintained in mammalian plasma

We will now turn to a brief consideration of studies on mammalian hearts in situ in which the vascular connections between the heart and other organs are partly or completely severed so that it is possible both to achieve some control over factors that affect myocardial function and to measure the rate of utilization of oxygen and other metabolic substrates The prototype preparation is of course the heart lung preparation but there are also various interesting modifications in which 1 or more organs of the body are excluded from the general circulation It was clearly stated by Starling and Visscher[9] that the classical heart lung preparation deteriorates over a period of several hours a decline which is reflected in both a decrease in contractility and in efficiency It is apparent that many factors may contribute to the decline of myocardial performance in the heart lung preparation For example a period of impaired coronary blood flow is almost inevitable and if prolonged will produce irreversible myocardial damage The heart lung preparation is deprived of sympathetic tone and probably undergoes progressive depletion of the cardiac sympathomimetic amines which are an important determinant of myocardial contractility Various substances produced by other organs of the body will not be available to the heart lung preparation and these may conceivably be of importance at the level of either energy production or of energy utilization Finally disturbances may be produced by the tubing of the extracorporeal part of the system and emboli may arise either from small clots or from bits of dried blood that form on the walls of the venous reservoir In fact, one cannot overemphasize what a poor performance the heart lung preparation renders compared to the intact heart (For interesting recent data see references 21 22 and 23)

Although it is therefore practically impossible to find one s way among the various difficulties inherent in these preparations 1 or 2 facts of interest stand out In the first place it becomes apparent from the results of several groups of investigators that myocardial performance is better when the liver and spleen are included in the circulation[2, 4, 6] In some studies the effect of liver and spleen appear to be primarily on the

metabolism of the heart,[4] whereas in others some factor from the liver and spleen seems to have a primary effect on myocardial contractility.[5, 6] The nature of such a substance isolated from hepatic venous blood after stimulation of the splenic nerve has been said by Schmier to be a polypeptide.[26] Recently Sayers' group has suggested that hormones from the adrenal cortex may delay the decline of performance seen in a heart lung preparation of the rat.[7] These findings, like those reviewed for isolated cardiac muscle, must be evaluated by asking why the heart lung preparation is depressed in the first place and whether the beneficial substances reported are important only in the experimental protocols or whether they have a general physiologic significance.

A new and encouraging note in the field of in situ heart preparations has been introduced by perfusing the coronary system of the experimental heart with blood from a healthy donor dog.[3, 8] This preparation which has been studied by Sarnoff and co-workers sometimes maintains a steady level of contractility for several hours. Therefore despite all the difficulties that we have enumerated it is possible for a denervated heart to maintain a fair level of functional capacity over a period of time, provided that vascular contact with a normal donor dog is maintained. One cannot say whether one of the beneficial effects of the donor animal is the maintenance at normal levels of some hypothetic cardiac active substance but at least this preparation might provide a basis for experimental investigation of the point.

It would be of great interest for example to determine cardioglobulin concentrations in such a preparation. We know in the case of the rat that an extracorporeal circuit that includes a filter with a large air blood interface is associated with rapid destruction of cardioglobulin C. A comparable destruction occurs in patients subjected to a period on a heart lung machine. Whereas there is a comparatively small change in cardioglobulin C concentration during the pumping period of 30 to 45 minutes we have found that there is a marked drop in cardioglobulin concentration during the first few hours after surgery. During the ensuing 24 hours considerable recovery occurs with cardioglobulin concentrations again approaching normal.

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Discussion

Dr DeHaan Dr Hoffman, I should like to ask a question—or 2 questions as a matter of fact First do you believe that this very interesting disturbance, apparently of the all or none response of the bundle or bundle branches negates Curtis and Travis s findings in 1951 of the all-or none response?

Dr Hoffman No I don t think our observations negate the findings of Curtis and Travis (*Am J Physiol* 165 173 1951) If a portion of the bundle or false tendon is stimulated and the action potential propagates in tissue that is fully repolarized conduction will be all-or none However if there are local differences in action potential duration and if for this reason propagation enters partially depolarized fibers decremental conduction of block may occur

Dr DeHaan May I go on to the second question? In your closing comments you mentioned a graded transition from conduction cells to typical ventricular cells I assume that you were referring to the electrophysiologic transition that is the gradual change from 1 type of action potential to another Or did you have some other evidence that the transition is really a graded one and not individual differences between cells in a fiber

Dr Hoffman I was thinking of both If you penetrate many fibers in the region of the junction you get action potentials that are typical of Purkinje fibers typical of ventricular muscle and also of every conceivable intermediate form Also I thought that Kugler and Parkins (*Anat Rec* 126 335 1956) among others had studied the histology of this region and shown at least 3 branches in the peripheral Purkinje fibers all in nice focus in the same section I think they called these branches A B and C they look as if a gradual progression were present Except for a few slides of our own their anatomic studies are what I had in mind

Dr Rhodin It is very satisfying to learn that our anatomic findings and Dr Weidmann s physiologic ones concerning the number of cell divisions and their role in the spread of excitation agree so well There is one bit of information I should like to add to what I said yesterday In comparing the intercalated discs in the myocardium and what I call the desmosome type that we find in the cells of the specific tissue as perceived in the bundle of His and its branches the surface of contact is increased at least by a factor of 50 so this again I think would explain that the impulse travels so much faster in the specific tissue

Dr Weidmann In which kind of cells is the increase—Purkinje or myocardial?

Dr Rhodin Purkinje fibers

Dr Paul F Cranefield (Brooklyn N Y) Dr Weidmann s work on the diffusion of potassium is very elegant and important The membrane at the intercalated disc is obviously there anatomically and ever since this has been known there has been a tendency for people to assume that the disc is a barrier to conduction Its anatomic presence does not of course prove that it has any electrophysiologic function

I feel that if the disc were to act as a direct impediment to conduction then it should have a high resistance to the diffusion of ions conversely if it did have a low resistance then it is hard to imagine how it could slow down propagation in any way There is in my opinion as of now no acceptable direct evidence that there is any such barrier to ionic movement

I have noticed in the last few years a sort of willingness almost a desire to assume that this disc is a barrier to conduction If we are forced to accept the disc as an impediment to conduction then we will be faced with reconstructing all the electrophysiology of the heart on a radically new basis I would urge all those who are not actively engaged in research bearing on this controversy to use caution and discretion in adopting this hypothesis as a possible explanation for their own observations

Chairman Brooks Of course there is another view also creeping in I think that

is, that the disc aids conduction I agree that this question of disc effect must be handled with discretion

The first item in the next discussion is a 4 minute motion picture film which will be presented by Dr Podolsky

Dr Podolsky Dr Winegrad and Dr Bianchi showed that calcium is made available to the muscle cell when it is stimulated The question then is what happens next? One way of finding out is to puncture the membrane with a micropipette and inject different solutions in the cell This technic was first used by Heilbrunn and Wiercynski (*J Cell & Comp Physiol* 29 15 1947) and later by Niedergerke (*J Physiol* 128 12 P 1955) and they got striking results by injecting calcium

Another preparation which is considerably more flexible was invented by the Japanese physiologist Natori (*Jikeikai M J* 1 119, 1954) He showed that the membrane of a single muscle fiber could be removed by microdissection and that the preparation was responsive to applied solution

I should like to show some experiments made with Natori s preparation which demonstrate that it responds to calcium but not to magnesium sodium or potassium

(Showing of the film with the following comments by Dr Podolsky)

The membrane has been removed from the fiber and the myofibrils are immersed in mineral oil An ordinary light miscroscope is used for observation The striations are quite obvious The pipettes are loaded with various solutions Drops can be formed at the tip of the pipette and then applied to the myofibril Since the pipettes are loaded with different solutions the responses can be compared

The first sequence shows a region where the membrane has been taken off and another where the membrane is intact The test solution contains calcium at 3 mM When drops are applied to the region *with* membrane there is no response Then if they are applied to the *stripped* region there is a contraction that is completely reversible

The next experiment shows that if calcium is omitted, there is no response Contraction is elicited only in that region of the myofibril which is in contact with the calcium solution There seems to be no spread of the contraction

The last sequence shows that, if calcium is replaced by magnesium there is no contractile response Also sodium and potassium at concentrations of 140 mM , do not trigger off the contractile mechanism

Chairman Brooks At Dr Weidmann's request I should like to recognize some people from Cleveland who are working on this problem

Dr T Hoshiko (Cleveland Ohio) I should like to draw attention to the work of Dr Rothschuh (*Arch ges Physiol* 225 238 1951) and to some work done with Drs Sperelakis and Berne (*Am J Physiol* 198 135 1960 *Am J Physiol* 198 531 1960, *Fed Proc* 19 108 1960 *Proc Soc Exper Biol & Med* 101 602 1959) that we feel show up some difficulties in the syncytial theory (1) Dr Rothschuh showed that in cardiac muscle depolarization upon injury will spread for only a short distance less than 1 mm In skeletal muscle such depolarization will gradually spread over the whole length of the muscle (2) In frog heart perfused with hypertonic solution quiescent cells with normal resting potentials were found adjacent to very active cells (3) The resistance of the single frog ventricular cell was high namely 12 megohms The resistance between 2 cell interiors was approximately double this value (4) We have induced unidirectional propagation in a predictable direction in frog ventricular strips this phenomenon cannot be easily explained in terms of a functional syncetium (5) We found that tissue DC resistance of heart muscle increased 7 fold when the interspace ion concentration was reduced by one tenth on the other hand in sartorius muscle where the cells extend almost the whole length of the muscle the resistance increase was only 2 to 3 fold

Finally as shown in figure 1 we measured the impedance of cat papillary muscle strips at various frequencies before (open stars)

and after (solid stars) intersp… tion The initial impedance at … equal to 1 These were compar… pedance of sartorius muscle b… cles) and after (solid circles… tonic sucrose for about 1 hour … in sucrose sartorius muscle … independent of frequency whi… cle impedance fell slightly at … quencies After soaking in su… muscle impedance rose 2 fold … independent of these frequ… th… other hand cardiac muscle … 10 fold at the low frequenc… tically at the higher frequ… higher frequencies a high resist… to be short-circuited by a capacit… nent

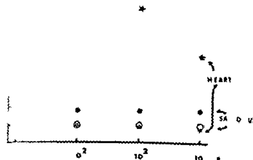


Figure 1

… pedance versus frequency in Tyrode's … ☆ in sucrose • ✶ Ordinate=im… …abscissa=frequency

These facts taken together appear to indicate the presence in cardiac muscle of transverse membranes of respectable resistance and capacitance which probably are the intercalated discs High resistance discs would make the functional syncytium theoretically inadmissible

Dr Weidmann I will be very brief I think that these membranes have some resistance and I am quite sure that they represent a capacity The point of the argument was really a quantitative one rather than a qualitative one I think we have to go privately through all of the data and see what can be done

Dr Eichna I guess I don t understand the structure of the intercalated discs Is the intercalated disc part of the same membrane that forms the wall of the cell or is it not?

Dr Weidmann It is part of the same membrane The membrane wrapping the side of the cell toward the extracellular space continues directly into the disc so I guess it is the same material But the functional significance of 2 apposed membranes may not be the same as that of 1 membrane in contact with the extracellular space After all the ionic composition might be different and this difference may change the resistance of the membrane

Dr Bing I should like to comment on Dr Hajdu s observation that the hypodynamic state is at first observed only at low frequencies but that later on further washing it occurs even at high frequencies

It is certainly true that the mechanical efficiency of the isolated heart is low We were unable to restore normal efficiency by adding plasma from normal dogs to the perfusion fluid We have ascribed the decline in mechanical efficiency of the isolated heart to depletion of catecholamines in the perfusion fluid This increase in efficiency occurs upon addition of small quantities of catecholamines and upon the inclusion of liver and spleen in the perfusion circuit However the changes in efficiency did not result from increased cardiac work but from a diminution in myocardial oxygen consumption It seems to me that it would be nice to have Dr Hajdu s cardioglobin defined more precisely

Dr Hajdu To deal with your second point first the addition of norepinephrine increases efficiency but does not restore cardiac contractility to normal As to the failure of fresh blood to restore contractility it is difficult by exchange transfusion methods to increase the concentration of certain substances to normal Our own experience with cardioglobulin bears on this point since we have found that in preparations which include an extracorporeal circuit the concen

tration declines rather rapidly and cannot be brought back by the exchange method

Dr Olson I should like to ask Dr Winegrad a question With regard to your very interesting calculation of molar ratios of Ca^{++} to actomyosin I take it you meant myosin and not actomyosin Is that correct?

Dr Winegrad These calculations are very gross and I will leave disagreement about the molecular weights to the biochemists The figure of 500 000 was used as an approximation of the actomyosin unit

Dr Olson But the measurements on actomyosin show that it is a much larger molecule Although actin G is 70 000, actin F is about $1\ 5 \times 10^6$ and the measurements on skeletal actomyosin show it to be an enormous molecule of the order of 20×10^6 in weight

Dr Winegrad That s right

Dr Olson The thing that occurred to me is that calcium introduced in vitro might activate myosin ATPase but in the intact fibril Perry (*Physiol Rev* 36 1 1956) has shown that magnesium is a more effective activator than calcium The question of myosin ATPase activation by ions is very interesting and still controversial Even the movie revealing an inotropic effect of added Ca^{++} which Dr Podolsky showed involves concentrations of calcium ions very much larger than the amount which you calculate move intracellularly during contraction Isn t that true?

Dr Winegrad There are 2 considerations in this respect First since uniform distribution of the calcium entering the cell during contraction is probably not immediately achieved regions with calcium concentrations higher than these calculations show will exist inside the cell transiently

Second, with reference to the specific values of calcium concentration at uniform distribution these values are of the same order that Dr Ebashi (unpublished data) has found will affect the action of his relaxing factor system on in vitro actomyosin preparations

Three experiments were performed in which the guinea pig atria were stimulated at 60/minute At this rate the atria could not maintain their contraction tension for 10 minutes without some decline A decline in calcium uptake per beat also occurred in these experiments These tissues have been irreversibly damaged and have suffered what one might call an acute type of failure, possibly related to the altered calcium metabolism

Dr Olson I should like to say one thing about the origin of the heart failure of reduced energy utilization All the evidence suggests that the oxidative process in this type of heart failure is normal A multiple etiology may be involved for example there may be a disturbance in some aspect of chemical mechanical coupling during contraction or other factors such as Dr Hajdu s plasma factor or a variety of steroids or the size and shape of the myosin molecule, may all be critical for the normal contractile cycle It may very well be that ultimately we can classify the heart failure of reduced energy utilization into several subcategories

Chairman Brooks I was hoping we could end the symposium in a blaze of light rather than in an increasing darkness [referring to lights which had just been turned on] Now that the lights have come on again our purpose is accomplished In closing I wish to thank the panel for their contribution to this meeting

The Pulsatile Beat of the Heart

I am obliged to conclude that in animals the blood is driven round in a circuit with an unceasing circular sort of movement that this is an activity or function of the heart which it carries out by virtue of its pulsations and that in sum it constitutes the sole reason for the heart s pulsatile movement —W Harvey *Movement of the Heart and Blood in Animals* Translated by N J Franklin Springfield Ill Charles C Thomas 1959 p 87

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tration declines rather rapidly and cannot be brought back by the exchange method

Dr Olson I should like to ask Dr Winegrad a question With regard to your very interesting calculation of molar ratios of Ca^{++} to actomyosin I take it you meant myosin and not actomyosin Is that correct?

Dr Winegrad These calculations are very gross and I will leave disagreement about the molecular weights to the biochemists The figure of 500 000 was used as an approximation of the actomyosin unit

Dr Olson But the measurements on actomyosin show that it is a much larger molecule Although actin G is 70 000 actin F is about 1.5×10^6 and the measurements on skeletal actomyosin show it to be an enormous molecule of the order of 20×10^6 in weight

Dr Winegrad That s right

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